EVALUATION OF MICROBIAL REDUCTIVE DECHLORINATION IN TETRACHLOROETHENE (PCE) DENSE NONAQUEOUS PHASE LIQUID (DNAPL) SOURCE ZONES

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Benjamin Keith Amos

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EVALUATION OF MICROBIAL REDUCTIVE DECHLORINATION IN TETRACHLOROETHENE (PCE) DENSE NONAQUEOUS PHASE LIQUID (DNAPL) SOURCE ZONES

Approved by:

Dr. Frank E. Löffler, Advisor
School of Civil and Environmental Engineering
Georgia Institute of Technology

Dr. Jim C. Spain
School of Civil and Environmental Engineering
Georgia Institute of Technology

Dr. Kurt D. Pennell
School of Civil and Environmental Engineering
Georgia Institute of Technology

Dr. Martial Taillefert
School of Earth and Atmospheric Sciences
Georgia Institute of Technology

Dr. Joseph B. Hughes
School of Civil and Environmental Engineering
Georgia Institute of Technology

Date Approved: July 3, 2007

I wish sometimes I could rewrite some chapters,
some paragraphs, some passages, some lines,
but it's those parts I'd change, those lines I'd cut,
those scenes that I'd refine,
those things that I would change
are the things that make the story
mine

Allen Levi

To Amanda, Mother, and Daddy
for their constant love and support

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I also owe a dept of gratitude to my parents because they have always believed in me. I would not be the man I am today without them, and I hope to live up to the truly amazing examples that they set everyday. I thank them for instilling in me the value of education, for teaching me the difference between right and wrong, for rooting my life in the belief that there is a higher calling and that we do have a sense of purpose, and for constantly demonstrating what hard work really is. These values are the core of my being and will take me far in life. I also thank my parents for letting me grow up on a farm; it is there that I gained my sense of wonder and appreciation for the natural world. I also believe that my experiences growing up gave me the ability to solve problems and trouble shoot, skills that are essential to being a good researcher. I could not have performed the research described in this dissertation without my love of the environment or my problem solving skills.

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1,1-DCE	1,1-dichloroethene
1-D	one-dimensional
2-D	two-dimensional
3-D	three-dimensional
ABI	Applied Biosystems
ATCC	American Type Culture Collection
BDI	Bio-Dechlor INOCULUM
bgs	below ground surface
BHQ	black hole quencher
BLAST	basic local alignment tool
bp	base pair
ca.	circa
cDNA	complementary DNA
CERCLIS	Comprehensive Environmental Response, Compensation, and Liability Information System
cis-DCE	*cis*-1,2-dichloroethene
CMC	critical micelle concentration
d	day
DCE	dichloroethene
DEPC	diethyl pyrocarbonate
DGGE	denaturing gradient gel electrophoresis
DMD	density modified displacement
DNA	deoxyribonucleic acid
DNAPL	dense nonaqueous phase liquid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
–dRn/dT	negative derivate of Rn with respect to T
DSM	Deutsche Sammlung von Mikroorganismen (German Collection of Microorganisms)
e^-	electron
EDTA	ethylenediamine tetraacetic acid
EPA	U.S. Environmental Protection Agency
ERH	electric resistive heating
FID	flame ionization detector
g	gravitational constant
GC	gas chromatograph
	percentage of nitrogenous bases in a DNA molecule which are either guanine or cytosine
HD	hexadecane
HP	Hewlett-Packard

HPLC	high performance liquid chromatography
hr	hour
i.d.	inside diameter
IFC	Integrated Field-Scale Subsurface Research Challenge
IFT	interfacial tension
ISCO	*in situ* chemical oxidation
K_h	Henry's law constants (dimensionless)
LC	low chloride
Mb	mega base pairs
MBT	molecular biological tool
MCL	maximum contaminant level
MISER	Michigan Soil Vapor Extraction Remediation
MLS	multilevel sampling
mol%	mole percent
mRNA	messenger RNA
MSR	molar solubilization ratio
mV	millivolts
n	number of samples or replicates
NAPL	nonaqueous phase liquid
ND	not detected or not determined
NPL	National Priorities List
NTC	no template control
o.d.	outside diameter
PCE	tetrachloroethene
PCR	polymerase chain reaction
POC	particulate organic carbon
ppb	parts per billion
psig	pounds per square inch gauge
PV	pore volume
qPCR	quantitative real-time PCR
RDase	reductive dehalogenase
redox	oxidation-reduction
Rn	reported fluorescent signal
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	reverse transcription
RT-qPCR	reverse transcription qPCR
SD	standard deviation
SDS	sodium dodecylsulfate
SEAR	surfactant enhanced aquifer remediation
sec	second
SEE	steam enhanced extraction
sp.	species (singular)
spp.	species (plural)

T	temperature
TCE	trichloroethene
TEAP	terminal electron-accepting process
TES	N-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid ($C_6H_{15}NO_6S$)
T_m	melting temperature
trans-DCE	*trans*-1,2-dichloroethene
U	unit (measure of enzymatic activity)
UTCHEM	University of Texas Chemical Compositional Simulator
VC	vinyl chloride
vol/vol	volume per volume
wt%	weight percent

a'_{ao}	specific interfacial area
a_{ao}	interfacial area
Bn	bioavailability number
C_a^{PCE}	PCE concentration in the bulk aqueous phase
$C_{ao}^{PCE\text{-}e}$	PCE aqueous phase concentration at equilibrium with organic phase PCE
J	net contaminant flux
k	pseudo first-order reaction rate (day^{-1})
k_{max}^{PCE}	maximum utilization rate for PCE
K_S^{PCE}	half-saturation constant for PCE
N	number of cells per mL
N_o	number of cells per mL initially or in the absence of contaminating DNA
R^2	coefficient of determination
t	time
X_o	cell titer (i.e., biomass concentration)

δ	boundary layer (film) thickness
κ^{PCE}	mass transfer coefficient of PCE from the organic phase to the bulk aqueous phase

SUMMARY

Chlorinated solvents, such as tetrachloroethene (PCE), are major groundwater contaminants that often persist as dense, nonaqueous phase liquids (DNAPLs) in subsurface environments. Dissolved-phase PCE plumes emanate from PCE-DNAPL source zones, which act as continuous sources of contamination for decades. Removal of DNAPL source zones is crucial to achieve lasting remedy of contaminated aquifers. Considerable research has focused on the development and demonstration of physical-chemical *in situ* remediation technologies (e.g., surfactant flushing, cosolvent flushing, chemical oxidation, thermal treatment) for DNAPL mass recovery from contaminated source zones. Although promising, none of the currently available physical-chemical remedies completely removes all DNAPL within source zones. Therefore, continued and persistent contaminant elution and plume formation occurs after application of physical-chemical treatments, often resulting in plume concentrations that still exceed regulatory limits.

Microbial reductive dechlorination (i.e., [de]chlororespiration) is emerging as a promising approach for the remediation of PCE-DNAPL source zones. In microbial reductive dechlorination, specialized bacteria obtain energy for growth from metabolic dechlorination reactions that convert PCE to trichloroethene (TCE), *cis*-1,2-dichloroethene (*cis*-DCE), vinyl chloride (VC), and finally to benign ethene. Microbial reductive dechlorination within PCE-DNAPL source zones was once thought to be infeasible due to the toxicity of free-phase PCE and high PCE concentrations to dechlorinating microorganisms. Recently, dechlorinating isolates and mixed consortia

were reported to dechlorinate PCE at or near saturated concentrations, suggesting that biologically enhanced (bioenhanced) DNAPL dissolution is possible. Laboratory experiments demonstrated that the microbial reductive dechlorination process can promote bioenhanced DNAPL dissolution. In bioenhanced dissolution, biological activity in proximity of the NAPL-water interface affects interphase mass transfer due to (i) the increase in the driving force for PCE dissolution as a result of reduced PCE concentrations in the aqueous phase, and (ii) the transformation of PCE to compounds with higher aqueous phase solubilities (e.g., DCEs).

For successful implementation of the microbial reductive dechlorination process to remediate DNAPL source zones, the mechanisms controlling microbial activity in the presence of DNAPL and the influence of microbial distribution on biologically-enhanced (bioenhanced) PCE dissolution must be elucidated. Synergistic effects of coupling physical-chemical remedies with biological activity, as observed in recent pilot-scale field demonstrations of surfactant and cosolvent (ethanol) flushing, also need further exploration as a potential strategy for PCE-DNAPL source zone treatment. The overall objective of this research, therefore, was to address key gaps in the scientific and engineering understanding of PCE-DNAPL source zone bioremediation. This work explored the contributions of the microbial reductive dechlorination process to PCE-DNAPL source zone remediation, either in isolation or as a polishing step for the removal of residual DNAPL remaining after application of surfactant enhanced aquifer remediation (SEAR), an emerging physical-chemical treatment. Specific objectives of this research and key findings of each objective are outlined below.

Objective 1: Evaluate the ability of microorganisms to dechlorinate in the presence of PCE-DNAPL and at high dissolved-phase PCE concentrations expected near/in DNAPL source zones

Experiments to address this objective were performed in batch cultures of *Sulfurospirillum multivorans*, *Desulfuromonas michiganensis* strain BB1, *Geobacter lovleyi* strain SZ, and *Desulfitobacterium* sp. strain Viet1. Key findings include:

- Despite recent evidence suggesting that microbial reductive dechlorination occurs at or near PCE saturation (0.9 – 1.2 mM), dechlorination was inhibited at PCE concentrations >540 μM for the tested isolates.
- In the presence of PCE-DNAPL:
  - Dechlorination occurred as long as PCE concentrations remained below inhibitory levels (<540 μM).
  - Under appropriate conditions, complete DNAPL dissolution was observed.
- These results:
  - Suggest that microorganisms incapable of dechlorinating at high PCE concentrations can enhance DNAPL dissolution.
  - Challenge the current paradigm that microbial activity at high PCE concentrations is necessary for bioenhanced DNAPL dissolution.
  - Enhance the understanding of microbial species that can potentially play significant roles in source zone bioremediation.

Objective 2: Assess the distribution and activity of key dechlorinating populations during bioenhanced PCE-DNAPL dissolution in continuous-flow column experiments

Experiments to address this objective were performed in continuous-flow column systems containing simulated PCE-DNAPL source zones. The column experiments were performed with two cultures: a pure PCE-to-*cis*-DCE dechlorinating isolate, *Sulfurospirillum multivorans*, and a PCE-to-ethene dechlorinating consortium, BDI-SZ, which contained multiple *Dehalococcoides* spp. and two PCE-to-*cis*-DCE dechlorinating populations (*Geobacter lovleyi* strain SZ and a *Dehalobacter* species). Key findings include:

- *Sulfurospirillum*, *Geobacter*, and *Dehalococcoides* populations were detected and active in NAPL source zones.
- Increases in dechlorinating populations coincided spatially with increased formation of daughter products.
- Greater than 4-fold cumulative enhancement of NAPL dissolution was observed.
- The distribution of relevant dechlorinating microbial populations directly affected dissolution enhancement.
- Complete conversion of PCE to ethene was not observed in the BDI-SZ column, suggesting that complete detoxification of PCE may not occur during source zone bioremediation.
- Bioenhanced dissolution only occurred when aqueous PCE concentrations were below inhibitory levels.

Objective 3: Determine the influence of Tween 80, a biodegradable surfactant commonly used in SEAR, on the microbial reductive dechlorination process

For microbial reductive dechlorination to be considered feasible following SEAR, the surfactant(s) utilized during SEAR should have little or reversible impact (i.e., toxicity, inhibition) on the microbial populations relevant for the detoxification process. The influence of Tween 80 (a nonionic, food-grade, biodegradable surfactant commonly used in SEAR) on the microbial reductive dechlorination process was evaluated in batch cultures. Key findings include:

- Tween 80 did not inhibit dechlorination by PCE-to-TCE or PCE-to-*cis*-DCE dechlorinators (e.g., *Geobacter lovleyi* strain SZ).
- Dechlorination by *Dehalococcoides* spp. was inhibited by Tween 80 in both pure and mixed cultures.
- The number of *Dehalococcoides* cells decreased exponentially during exposure to Tween 80.
- The dechlorination activity of *Dehalococcoides* spp. was recovered after exposure to Tween 80 (i.e., the effect of Tween 80 was reversible).
- The limited and reversible impact of Tween 80 on key dechlorinators supported the feasibility of a treatment train approach of SEAR followed by microbial reductive dechlorination to more completely remediate PCE-DNAPL source zones.

Objective 4: Design and optimize quantitative real-time PCR (qPCR) protocols to detect and enumerate key dechlorinating populations

The application of molecular biological tools (e.g., qPCR) to assess and monitor bioremediation has greatly improved the ability of scientists and engineers to establish cause-and-effect relationships between anaerobic microbial processes and contaminant detoxification. The present work describes the design, optimization, and application of new molecular tools (i.e., qPCR protocols) to specifically detect and quantify *Sulfurospirillum multivorans* and *Geobacter lovleyi* strain SZ. The use of these new molecular tools was critical in exploring the distribution of these environmentally-relevant dechlorinators within DNAPL source zones (Objective 2). An additional study evaluated (i) the environmental distribution of *Geobacter lovleyi* strain SZ and (ii) the ability of this versatile organism to respond to *in situ* biostimulation at a mixed waste site in Oak Ridge, Tennessee. Key findings from the additional study include:

- *Geobacter lovleyi* strain SZ is likely a relevant contributor to chlorinated ethene detoxification within diverse environments since strain SZ-like organisms were detected in:
  - several PCE-dechlorinating enrichment cultures,
  - the KB-1 culture, a widely used bioaugmentation consortium, and
  - environmental samples from chlorinated ethene-impacted aquifers.
- Strain SZ responded to *in situ* biostimulation at the mixed waste site in Oak Ridge, Tennessee, a critical characteristic for successful implementation of bioremediation approaches.

Objective 5: Explore the effects of oxygen on *Dehalococcoides* viability and biomarker quantification

Strict anaerobic *Dehalococcoides* strains play critical roles in detoxification of chlorinated ethenes, and molecular tools that target *Dehalococcoides* DNA and RNA biomarkers are important in monitoring and assessing bioremediation (including source zone bioremediation). These strict anaerobes may be exposed to oxygen during bioremediation, but the effect(s) of oxygen on *Dehalococcoides* activity and biomarker quantification are unknown. Therefore, experiments were performed with batch cultures of a PCE-to-ethene dechlorinating consortium (Bio-Dechlor INOCULUM; BDI) to explore the effects of oxygen on *Dehalococcoides* viability and to evaluate the resolution of current PCR-based tools to distinguish active, dechlorinating cells from inactive, oxygen-exposed cells. Key findings include:

- Quantifiable effects on *Dehalococcoides* activity following oxygen exposure were observed.
- Several lines of evidence suggest that, of the three *Dehalococcoides* strains in BDI, only strain FL2 (a TCE-to-VC dechlorinator) survived oxygen exposure while VC-dechlorinating strains (strain GT and strain BAV1) did not survive oxygen exposure.
- The current PCR-based tools used to detect and quantify *Dehalococcoides* DNA and RNA biomarkers did not prove useful in distinguishing viable, dechlorinating cells from inactive and irreversibly-inhibited cells, suggesting limitations in the ability of these molecular biological tools to infer cell viability and activity.
- New molecular tools are needed to complement existing technologies to improve the application of biomarker analysis in site assessment and bioremediation monitoring.

The findings of this research advance the scientific understanding of the microbial reductive dechlorination process and are relevant to environmental remediation practitioners. The results of this study will aid in the design and successful implementation of PCE-DNAPL source zone bioremediation. The gains made in understanding the complex interactions and processes involved in microbial reductive dechlorination and bioenhanced PCE-DNAPL dissolution will further promote source zone bioremediation as a viable technology for lasting and cost-effective remediation of DNAPL-impacted aquifers.

CHAPTER 1

INTRODUCTION

1.1 Background

Chlorinated solvents, such as tetrachloroethene (PCE), are common groundwater contaminants that are frequently encountered as dense nonaqueous phase liquids (DNAPLs) in aquifer formations (*1*). Subsurface environments that contain DNAPL, either as residual ganglia or pools, represent long-term threats to environmental and public health since contaminant elution from these source zones can occur for decades (*2*). Several physical-chemical *in situ* remediation technologies (e.g., surfactant flushing, chemical oxidation, thermal treatment) have been developed and employed for treatment of DNAPL source zones (i.e., the DNAPL-containing origin of the plume) (*1*). Although a significant amount (60-90%) of source zone contaminant mass can be removed or destroyed using the currently available physical-chemical technologies, near-term environmental risks may not be significantly reduced since post-treatment contaminant concentrations emanating from treated DNAPL source zones can still exceed regulatory limits (*3*).

Metabolic microbial reductive dechlorination (i.e., [de]chlororespiration) has recently emerged as a promising approach for the remediation of chlorinated ethene dissolved phase plumes emanating from DNAPL source zones. In metabolic microbial reductive dechlorination, specialized bacteria obtain energy for growth from metabolic dechlorination reactions (see Figure 1.1). Several microbial isolates transform PCE to

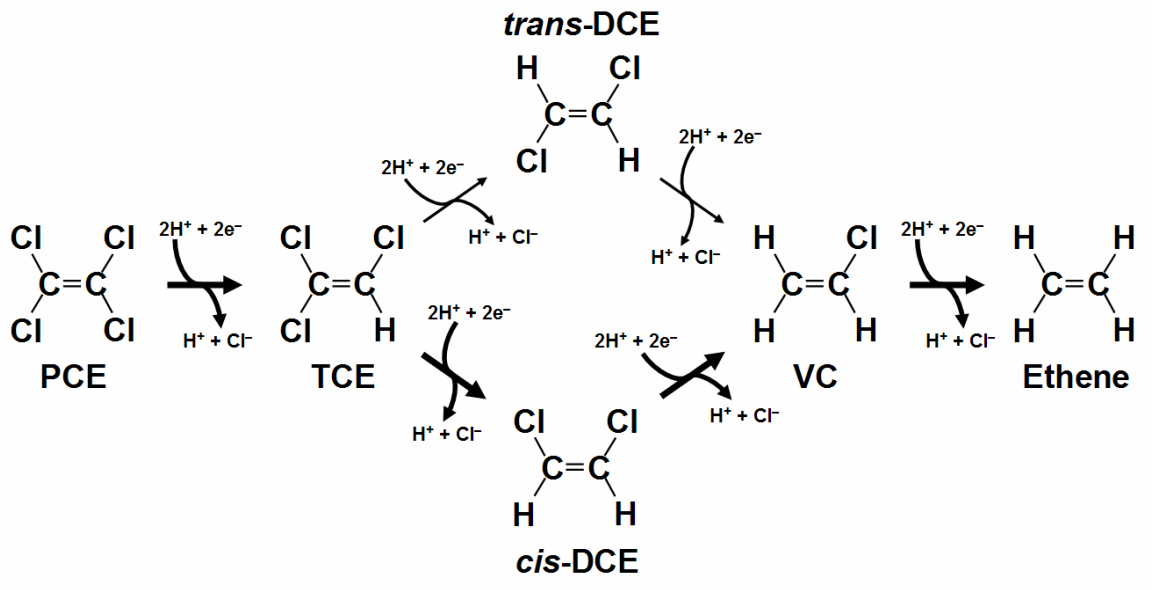

Figure 1.1 Microbial reductive dechlorination of PCE to ethene. The thick arrows indicate the predominate pathway (adapted from Löffler and Edwards (*4*)).

cis-1,2-dichloroethene (*cis*-DCE) (e.g., *Desulfuromonas* spp. (*5,6*), *Sulfurospirillum* spp. (*7,8*), *Geobacter lovleyi* strain SZ (*9*), *Desulfitobacterium* spp. (*10,11*), and *Dehalobacter* spp. (*12,13*)), but only some members of the *Dehalococcoides* group have been shown to dechlorinate beyond *cis*-DCE to vinyl chloride (VC) and benign ethene (summarized in (*4*)). None of the *Dehalococcoides* isolates grow with all chlorinated ethenes as electron acceptors (*4*), and efficient and complete dechlorination of PCE to ethene depends on the presence of multiple dechlorinating populations. Anaerobic bioremediation (i.e., biostimulation and bioaugmentation) utilizing the microbial reductive dechlorination process has been successfully implemented in the field to contain and treat chlorinated ethene plumes (*4*). Plume containment, however, does not reduce overall remediation time since the rate of contaminant dissolution from the DNAPL source zone remains unchanged.

Bioremediation within PCE-DNAPL source zones has recently been proposed as a source zone treatment technology and has the potential to reduce source zone longevity. Microbial reductive dechlorination within PCE-DNAPL source zones was once thought to be infeasible due to the toxicity of free-phase PCE and high PCE concentrations to dechlorinating microorganisms (*5,7,8,13*). Recently, dechlorinating isolates and mixed consortia were reported to dechlorinate PCE at or near saturated concentrations (*6,11,14-20*), suggesting that biologically enhanced (bioenhanced) DNAPL dissolution is possible. Laboratory experiments suggested that the microbial reductive dechlorination process could result in bioenhanced DNAPL dissolution (*16-21*). In bioenhanced dissolution, biological activity in proximity of the NAPL-water interface affects interphase mass transfer due to (i) the increase in the driving force for PCE dissolution as a result of

reduced PCE concentrations in the aqueous phase, and (ii) the transformation of PCE to compounds with higher aqueous phase solubilities (e.g., DCEs) (*1*). Although microbial reductive dechlorination is a promising technology for DNAPL source zone remediation, many questions remain regarding the effectiveness and implementation of this treatment strategy (e.g., see references (*1,17-23*)).

1.2 Thesis Rationale

For the microbial reductive dechlorination process to be widely accepted as a suitable option for source zone remediation, mechanisms controlling microbial activity in the presence of DNAPL and the influence of microbial distribution on bioenhanced dissolution must be elucidated. Synergistic effects of coupling physical-chemical remedies with biological activity, as observed in pilot-scale field demonstrations of surfactant and cosolvent (ethanol) flushing (*24,25*), also need further study as a potential strategy for PCE-DNAPL source zone treatment. The overall objective of this research, therefore, was to address key gaps in the scientific and engineering understanding of PCE-DNAPL source zone bioremediation. This work explored the contributions of the microbial reductive dechlorination process to PCE-DNAPL source zone remediation, either in isolation or as a polishing step for the removal of residual DNAPL remaining after application of surfactant enhanced aquifer remediation (SEAR), an emerging physical-chemical treatment. Specific objectives of this research, the results of which are detailed in Chapters 3-8, and the rationale for each objective are outlined below. A review of relevant literature is presented in Chapter 2, and overall conclusions resulting

from this work, as well as recommendations for future research, are presented in Chapter 9.

1.2.1 Objective 1: Evaluate the Ability of Microorganisms to Dechlorinate in the Presence of PCE-DNAPL and at High Dissolved-Phase PCE Concentrations Expected Near/In DNAPL Source Zones

Activity of dechlorinating microorganisms at high, saturated PCE concentrations expected near the NAPL-water interface and frequently encountered in PCE-DNAPL source zones is considered necessary for bioenhanced PCE-DNAPL dissolution. Many studies report contradictory evidence regarding the ability of dechlorinating isolates and consortia to dechlorinate at saturated, dissolved-phase PCE concentrations (*5-8,13-21*). Taken together, no definitive conclusions can be drawn regarding the effectiveness of the microbial reductive dechlorination process as a viable source zone treatment technology. Chapter 3 details experiments that account for the described discrepancies and discusses resulting implications for PCE-DNAPL source zone bioremediation.

1.2.2 Objective 2: Assess the Distribution and Activity of Key Dechlorinating Populations during Bioenhanced PCE-DNAPL Dissolution in Continuous-Flow Column Experiments

Several batch, one-dimensional (1-D) column, and two-dimensional (2-D) sand box experiments have been conducted to evaluate microbial reductive dechlorination performance and bioenhanced dissolution in simulated PCE-DNAPL source zones (*16-21*). These studies suggest dechlorination activity in the vicinity of PCE-DNAPL. While these initial results are promising, the activity of relevant microbial species within DNAPL source zones and the effect(s) of microbial distribution on dissolution

enhancement remain poorly understood. Therefore, 1-D, continuous-flow column experiments were performed to evaluate bioenhanced dissolution and microbial distribution in the immediate vicinity of a PCE-DNAPL source zone. As presented in Chapter 4, column experiments explored bioenhanced dissolution with a pure PCE-to-*cis*-DCE dechlorinating isolate, *Sulfurospirillum multivorans*. *S. multivorans* was selected for evaluation in the 1-D column experiments due to its ease of handling, robust growth characteristics, and the fact that it was observed to dechlorinate in the presence of PCE-DNAPL (Objective 1, Chapter 3). As presented in Chapter 5, an additional column experiment was undertaken with a PCE-to-ethene dechlorinating consortium that contained multiple *Dehalococcoides* spp. as well as two PCE-to-*cis*-DCE dechlorinating populations (*Geobacter lovleyi* strain SZ and a *Dehalobacter* species). The dechlorinating consortium was utilized for the following reasons: efficient and complete dechlorination of PCE to ethene depends on the presence of multiple dechlorinating organisms, dechlorinating consortia are often used as bioaugmentation inocula (*26,27*), and complex microbial communities dominate subsurface environments at contaminanted sites. Chapters 4 and 5 discuss insights into the relationships between microbial distribution and dissolution enhancement as well as current shortcomings of achieving complete PCE dechlorination to ethene during PCE-DNAPL source zone bioremediation.

1.2.3 Objective 3: Determine the Influence of Tween 80, a Biodegradable Surfactant Commonly used in SEAR, on the Microbial Reductive Dechlorination Process

Recent field studies have indicated synergistic effects of coupling microbial reductive dechlorination with physical-chemical remediation (e.g., surfactant flushing) of

DNAPL source zones (*24,25*). Such synergy may overcome the challenges associated with each technology when used in isolation. In a staged treatment scenario, the physical-chemical remedy removes significant contaminant mass, and, in case of surfactant or co-solvent flushing, delivers electron donors that may stimulate microbial reductive dechlorination activity (*24,25,28*). Hence, in this sequential approach, reductive dechlorination acts as a polishing step that detoxifies residual contaminants, thereby reducing contaminant mass flux and controlling long-term plume development. For coupled remediation of PCE-DNAPL source zones to be considered feasible, the physical-chemical remediation strategy should have little or reversible impact (i.e., toxicity, inhibition) on the microbial populations relevant for the detoxification process. Observations from previous studies (*25,29,30*) are contradictory and suggest that the effects of Tween 80, a biodegradable surfactant employed in source zone remediation (*31,32*), on the microbial reductive dechlorination process are insufficiently explored. To clarify these discrepancies, experiments were performed to determine the influence of Tween 80 on key dechlorinating bacteria and explore the feasibility of a "treatment train" approach of SEAR followed by microbial reductive dechlorination (Chapter 6).

1.2.4 Objective 4: Design and Optimize Quantitative Real-Time PCR (qPCR) Protocols to Detect and Enumerate Key Dechlorinating Populations

The application of molecular tools (e.g., quantitative real-time PCR [qPCR]) to assess and monitor bioremediation has greatly improved the ability of scientists and engineers to establish cause-and-effect relationships between microbial processes and contaminant detoxification (*4,33*). Use of these diagnostic tools, therefore, has gained increased acceptance with both regulators and environmental practitioners (*4,33*).

Molecular tools for sensitive, specific detection and quantification of many dechlorinating organisms (e.g., *Dehalococcoides* spp.) have been designed and are offered commercially (e.g., see references (*34-37*)). Such tools have not been developed for quantitative detection of two environmentally-relevant, PCE-to-*cis*-DCE dechlorinating organisms: *Sulfurospirillum multivorans* and *Geobacter lovleyi* strain SZ. The design, optimization, and application of qPCR protocols to detect and enumerate these dechlorinating species are described in Chapter 4 (*S. multivorans*) and Chapters 5 and 7 (*G. lovleyi*). In Chapters 4 and 5, the designed tools were used to assess microbial distribution within a simulated PCE-DNAPL source zone (see Objective 2). The results of Chapter 5 demonstrated an important role of *G. lovleyi* in bioenhanced DNAPL dissolution. Therefore, additional studies, presented in Chapter 7, evaluated (i) the environmental distribution (biogeography) of *G. lovleyi* and (ii) the ability of this versatile organism to respond to *in situ* biostimulation at a mixed waste site (Integrated Field-Scale Subsurface Research Challenge [IFC] site in Oak Ridge, Tennessee). The new detection and quantification tools add to the armamentarium of molecular tools available to (i) scientists to study the ecophysiology of these dechlorinating organisms and (ii) remedial managers for site assessment and bioremediation monitoring.

1.2.5 Objective 5: Explore the Effects of Oxygen on *Dehalococcoides* Viability and Biomarker Quantification

Strict anaerobic *Dehalococcoides* spp. play a critical role in detoxification of chlorinated contaminants (e.g., chlorinated ethenes) (*4*), and molecular tools that target *Dehalococcoides* DNA and RNA biomarkers are important in monitoring and assessing bioremediation (*4,33*). *Dehalococcoides* spp. are fastidious bacteria, possibly due to

unknown nutritional requirements and/or sensitivity to redox conditions and oxygen (*38,39*). *Dehalococcoides* organisms may be exposed to oxygen during transport and subsurface injection of *Dehalococcoides*-containing bioaugmentation cultures, subsurface delivery of electron donor(s) or other remedial solutions, and infiltration of oxygenated groundwater (e.g., rain events). The effect(s) of oxygen on *Dehalococcoides* activity and biomarker quantification are unknown. Chapter 8 details experiments that (i) explored the effects of oxygen on *Dehalococcoides* viability and (ii) evaluated the resolution of current PCR-based assays to distinguish active, dechlorinating from inactive, oxygen-exposed cells. The results of these studies are relevant to the bioremediation field, including the application and monitoring of microbial reductive dechlorination for PCE-DNAPL source zone treatment.

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CHAPTER 2

LITERATURE REVIEW

2.1 Chapter Overview

This chapter provides a brief review of relevant literature pertaining to tetrachloroethene (PCE) contamination, microbial reductive dechlorination, and dense nonaqueous phase liquid (DNAPL) source zone remediation. The first section details the extent and sources of chlorinated solvent contamination. This section is followed by a description of DNAPLs and DNAPL source zone characteristics. Current physical-chemical remediation technologies for DNAPL source zone treatment are then reviewed. The next sections provide information about microbial reductive dechlorination and bioenhanced PCE-DNAPL dissolution. Field evidence for the stimulatory and complementary effects of physical-chemical remedies on subsequent microbial reductive dechlorination is then reviewed. Finally, a brief description of molecular biological tools used to assess and monitor key microbial players in contaminant detoxification is provided. Additional literature related to the specific objectives outlined in Chapter 1 is further reviewed in Chapters 3-8.

2.2 Chlorinated Ethenes in the Environment

Many halogenated compounds are produced naturally by biotic or abiotic means, and, therefore, have been present in the environment for a long time, possibly billions of

years (*1-6*). Since the Industrial Revolution the production of halogenated compounds, chiefly chlorinated ones, has increased dramatically as diverse applications were discovered (*5*). For example, chlorinated solvents like PCE and trichloroethene (TCE) have been used extensively in dry cleaning operations and degreasing applications since the 1930s (*7,8*). PCE and TCE were often preferred over other organic solvents since they have superior solvent capabilities, are nonflammable and chemically stable, and were once believed to be benign (*7*). Due to frequent use, improper disposal practices, and accidental releases, widespread subsurface contamination by chlorinated ethenes has occurred (*7-10*). In the environment, abiotic or biotic transformation of PCE and TCE can result in accumulation of toxic daughter products, including *cis*-1,2-dichloroethene (*cis*-DCE), *trans*-1,2-dichloroethene (*trans*-DCE), and vinyl chloride (VC) (*5*). Taken together, chlorinated ethenes are one of the most common groups of groundwater contaminants in the industrialized world (see references (*8-10*) and as documented in the Comprehensive Environmental Response, Compensation, and Liability Information System [CERCLIS] database [http://cfpub.epa.gov/supercpad/cursites/srchsites.cfm]). For example, PCE and TCE have been detected at >50% of the 1,430 National Priorities List (NPL) sites identified by the U.S. Environmental Protection Agency (EPA) (*11,12*). Polychlorinated ethenes have stringent regulatory standards since they are toxic and suspected carcinogens (*11,12*). The current drinking water maximum contaminant levels (MCLs) for PCE, TCE, *cis*-DCE, and *trans*-DCE are 5 ppb, 5 ppb, 70 ppb, and 100 ppb, respectively (U.S. EPA; http://www.epa.gov/safewater/contaminants). VC is a proven human carcinogen with an even lower MCL of 2 ppb ((*13*) and http://www.epa.gov/safewater/contaminants). A list of properties of chlorinated ethenes

and their MCLs is presented in Table 2.1. PCE and TCE are challenging to remediate due to their volatility, relatively low aqueous solubility, hydrophobicity, retardation relative to groundwater flow, and ability to form separate organic phases in the subsurface (*7*).

2.3 Dense Nonaqueous Phase Liquid (DNAPL) Source Zones

PCE and TCE are typically released into the environment as organic liquids (either in pure form or as part of multi-component mixtures) (*8,10*). Since pure (neat) liquids of PCE and TCE are hydrophobic, sparingly soluble, and more dense than water (see Table 2.1), these compounds persist as distinct organic phases (i.e., dense nonaqueous phase liquids [DNAPLs]) in subsurface environments (*7,10*). An idealized DNAPL source zone (i.e., the DNAPL-containing origin of a dissolved-phase contaminant plume) is depicted in Figure 2.1. After a spill event or improper disposal, PCE and TCE DNAPLs migrate downward in the subsurface and penetrate below the water table into the water-saturated zone (*14*). During migration, the organic phase separates into isolated, immobile ganglia (residual DNAPL) due to capillary forces in the porous media (*15*). In addition, DNAPLs may continue to migrate until reaching a low permeability layer/capillary barrier (confining layer or lens) where DNAPLs tend to "pool" in high saturation zones and migrate laterally (*15*). In DNAPL pools, DNAPLs are connected through multiple soil pores (*16*). DNAPL chemical properties (e.g., viscosity, interfacial tension) and characteristics of the porous medium (e.g., wettability,

Table 2.1 Properties of chlorinated ethenes and ethene and their maximum contaminant levels (MCLs).

Compound	Molecular Weight	Density (g/mL) [a]	Aqueous Solubility (mg/L)	K_h [b]	MCL [c] (ppb)
PCE	165.83	1.625	200 [d]	0.723	5
TCE	131.39	1.462	1100 [e]	0.392	5
cis-DCE	96.94	1.248	3500 [f]	0.167	70
trans-DCE	96.94	1.257	6300 [f]	0.384	100
1,1-DCE [g]	96.94	1.214	400 [f]	1.069	7
VC	62.49	– [h]	2700 [e]	1.137	2
Ethene	28.05	– [h]	131 [e]	7.24	– [i]

[a] For pure (neat) liquids, as reported in Huling and Weaver (*15*)
[b] K_h, Henry's law constants (dimensionless) of chlorinated ethenes (Gossett (*17*)) or ethene (Coleman et al. (*18*))
[c] Maximum contaminant levels; U.S. EPA, http://www.epa.gov/safewater/contaminants
[d] Perry et al. (*19*)
[e] From Yaws (*20*), as reported in Yang and McCarty (*21*)
[f] As reported in Huling and Weaver (*15*)
[g] 1,1-dichloroethene
[h] These compounds are gases at environmentally relevant temperatures
[i] Ethene is not toxic and therefore does not have an EPA-mandated MCL

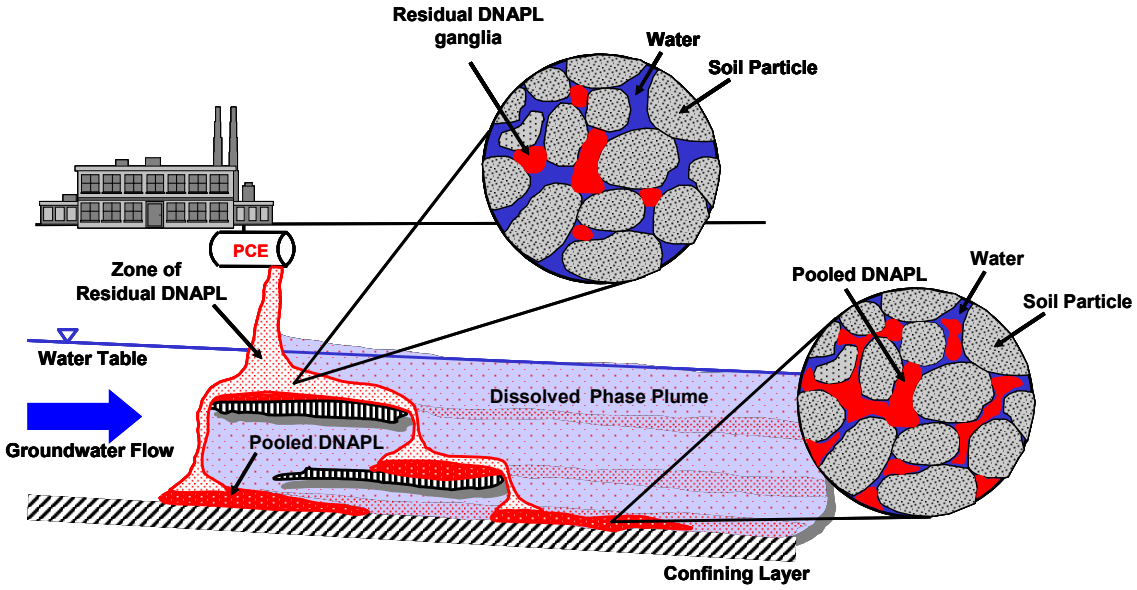

Figure 2.1 Idealized DNAPL source zone showing residual DNAPL ganglia, pooled DNAPL, and the formation of a dissolved-phase contaminant plume emanating from the source zone.

heterogeneity) help determine the specific behavior of NAPL infiltration and the resulting DNAPL saturation distribution (i.e., the source zone architecture) (*14*).

Due to slow contaminant dissolution from DNAPLs, source zones act as continuous sources of dissolved-phase contamination for decades (possibly centuries) and can contaminate large volumes of groundwater (*22,23*). DNAPL dissolution in aquifer formations, which is often rate limited by mass transfer from the organic to the aqueous phase (*24,25*), is a complex process that depends on many variables, including the source zone architecture, DNAPL interfacial area, porous media heterogeneity, and groundwater flow characteristics (*22,25-27*). A simplified representation of mass transfer from PCE-DNAPL to the aqueous phase is shown in Figure 2.2, where $C_{ao}^{PCE\text{-}e}$ is the PCE concentration in the aqueous phase at equilibrium with the organic phase, C_a^{PCE} is the PCE concentration in the bulk aqueous phase, and δ is the boundary layer (film) thickness. PCE-DNAPL dissolution is often described using a unidirectional linear driving force approximation (*24,25,28,29*), as shown in Equation 2.1:

$$J = \kappa^{PCE} a_{ao} (C_{ao}^{PCE\text{-}e} - C_a^{PCE}) \tag{2.1}$$

where J is the net contaminant flux (M/T), κ^{PCE} (L/T) is the mass transfer coefficient for PCE through the aqueous phase boundary layer, and a_{ao} (L^2) is the interfacial area between the aqueous and organic phases. PCE dissolution will proceed as long as $C_{ao}^{PCE\text{-}e} \geq C_a^{PCE}$, and many remedial technologies try to increase dissolution rates by decreasing C_a^{PCE} or increasing $C_{ao}^{PCE\text{-}e}$. Empirical correlations have been developed to predict mass transfer coefficients and contaminant dissolution based on groundwater flow and aquifer material characteristics (e.g., (*24*)). Although many of the correlations predict equilibrium aqueous phase contaminant concentrations at the local scale under

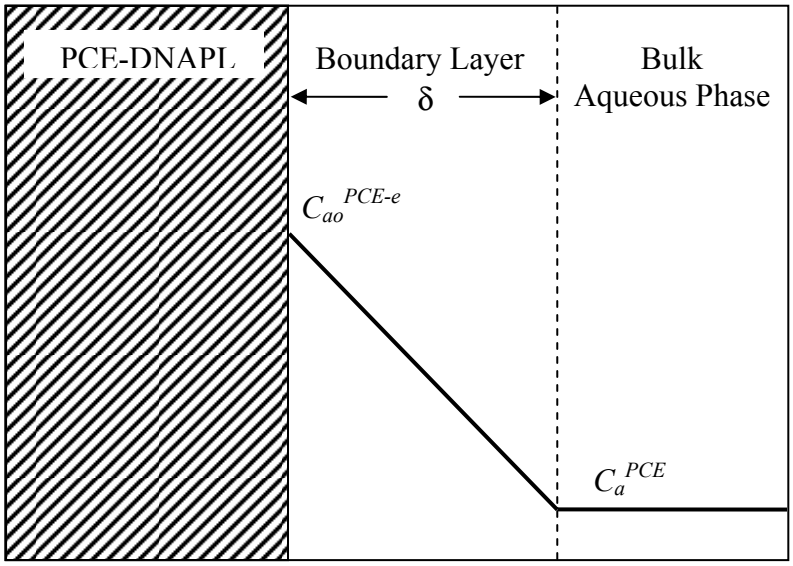

Figure 2.2 Simplified representation of mass transfer from PCE-DNAPL to the bulk aqueous phase (modified from Suchomel (*30*)).

most environmental conditions (e.g., (*24,25*)), contaminant concentrations are generally significantly lower than equilibrium solubility in dissolved-phase plumes due to dispersive transport and dilution (*15*). Such processes are also relevant in source zones with heterogeneous DNAPL distributions (*30*).

Containment of dissolved-phase contaminant plumes emanating from DNAPL source zones is technically feasible (*10,23*). Traditional approaches for hydraulic containment (e.g., pump-and-treat) are inefficient and expensive. For example, when considering pump-and-treat as the sole remedial action, the life cycle costs for cleanup of all U.S. DNAPL contaminanted sites are estimated to be between $50 and $100 billion (*23*). Given (i) the extent of DNAPL contamination (*23*), (ii) the longevity of DNAPL sources (*22*), (iii) the technical infeasibility of pump-and-treat DNAPL remediation to achieve regulatory goals within acceptable timeframes and costs (*10*), and (iv) the importance of subsurface aquifers as drinking water sources (*31*), alternative and innovative source zone remediation technologies are needed for source zone treatment.

2.4 Physical-Chemical Source Zone Remediation Technologies

This section presents a brief summary of select *in situ* physical-chemical source zone remediation technologies, including surfactant enhanced aquifer remediation (SEAR), cosolvent flushing, chemical oxidation, and thermal treatment. These innovative, emerging technologies, often referred to as “aggressive” or “active” source zone depletion technologies, can remove significant amounts of contaminant mass, reduce source zone longevity, and decrease near- and long-term environmental and health

risks (*8,10*). This section concludes with a discussion of the benefits and current shortcomings of physical-chemical remedies. Further information about the application of physical-chemical strategies for source zone treatment can be found in references (*8,10,15,23*).

2.4.1 Surfactant and Cosolvent Flushing

Due to the chemical properties of surfactants (e.g., soaps and detergents) and cosolvents (alcohols), these compounds increase contaminant aqueous phase solubility (solubilization = miscible displacement) and/or lower the interfacial tension (IFT) of the NAPL-water system (mobilization = immiscible displacement) (*8*). Solubilization and mobilization processes can result in contaminant mass removal from the subsurface as source zones are flushed with surfactants or cosolvents (*8*). Process specific mechanisms of surfactant and cosolvent flushing are discussed in Sections 2.4.1.1 and 2.4.1.2, respectively. In order for effective contaminant removal, the surfactant or cosolvent solution needs to efficiently contact DNAPL. That is, the flushing strategy should ensure a "good sweep efficiency" of the source zone (*8*). Achieving such contact may be difficult to obtain in heterogeneous systems due to reduced flow in low-permeability zones or preferential (bypassing) flow. One disadvantage of surfactant and cosolvent flushing is that the contaminants are not transformed to nontoxic compounds *in situ*; therefore, additional above ground processing is required. Surfactant and cosolvent flushing may also result in unintended DNAPL migration into deeper and/or previously uncontaminated aquifer regions; such unintended DNAPL mobilization can potentially be

alleviated by careful system design or *in situ* modifications of NAPL characteristics (e.g., density modified displacement [DMD] (*32,33*)) (*8,22*).

2.4.1.1 Surfactant Enhanced Aquifer Remediation (SEAR). Surfactants are surface active agents that preferentially accumulate at interfaces or surfaces of multiphase systems (*8*). Surfactants possess both hydrophilic and lipophilic (hydrophobic) moieties (*34*), as illustrated in Figure 2.3 for the nonionic, food-grade surfactant Tween 80 (polyoxyethylene [20] sorbitan monooleate; average molecular weight of 1,310 g/mole (*35*); critical micelle concentration [CMC] of 13 mg/L at 25ºC (*36*)). The hydrophobic moiety is often composed of a long chain alkane, while the hydrophilic region can vary from anionic, cationic, zwitterionic, or nonionic (*37*). Anionic (e.g., Aerosol MA, Steol CS-330) and nonionic surfactants (e.g., Tween 80, Brij 97, Triton X-100) have received the most attention for aquifer remediation. Surfactant selection depends on many variables, including cost, the intended mechanism of contaminant recovery (solubilization or mobilization), and subsurface characteristics (e.g., high organic content soils may be incompatible with some surfactants due to losses via sorption) (*37*). Use of readily biodegradable, nontoxic surfactants also minimizes concerns over the fate of unrecovered surfactant (*22*).

Application of surfactants for source zone remediation (i.e., surfactant enhanced aquifer remediation [SEAR]) can be divided into two broad classifications: miscible displacement (solubilization) or immiscible displacement (mobilization). In solubilization, the aqueous phase solubility of hydrophobic organic contaminants is substantially increased by application of surfactants at concentrations above their critical

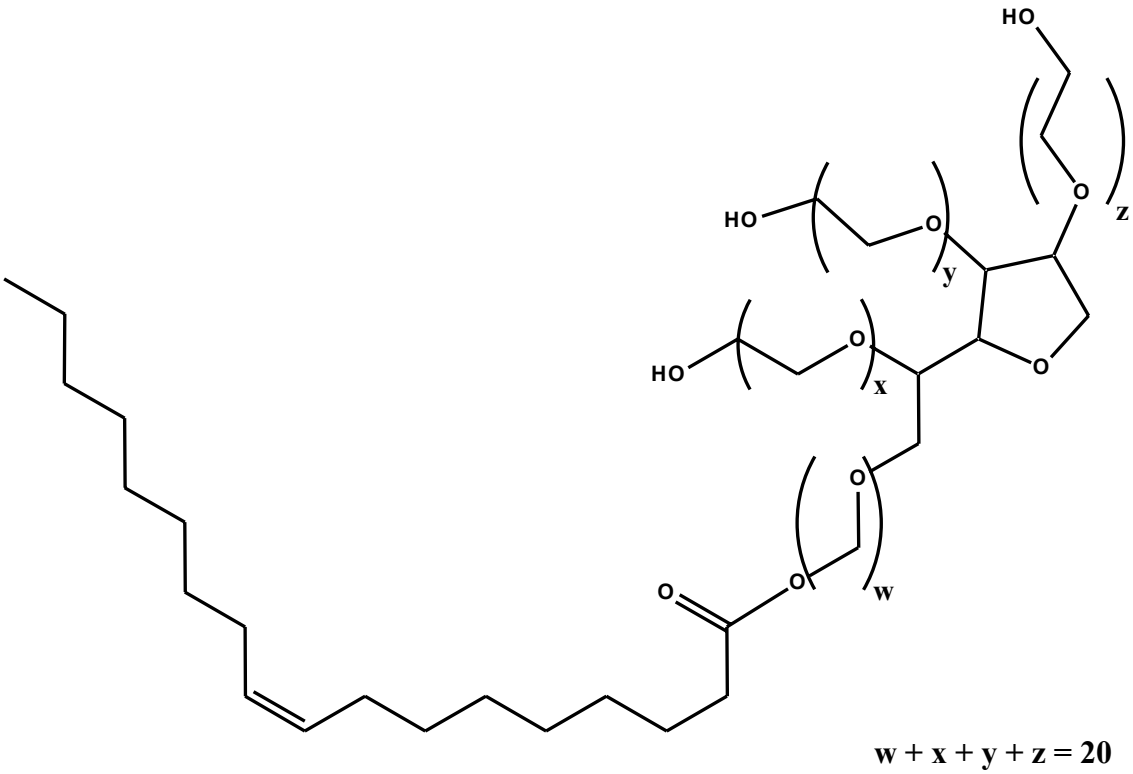

Figure 2.3 Structure of the nonionic, food-grade surfactant Tween 80.

micelle concentration (CMC) (reviewed in references (*34,35,37*)). When the aqueous phase concentration of a surfactant is at or above the CMC, surfactants form micelles (aggregates) containing 50 or more surfactant molecules (*8*). Micelle formation occurs due to the self association of lipophilic moieties of surfactant monomers with each other, resulting in surfactant aggregates with a hydrophobic core surrounded by a hydrophilic shell (*34*). Organic contaminants preferentially partition into micelles, which act as hydrophobic sinks, thereby raising the amount of the organic compound that can exist in the aqueous phase (*8,34*). As the number of micelles in solution increases, the apparent solubility of organic contaminants also increases (*8*). Therefore, surfactants are typically applied at concentrations significantly above their CMCs to maximize contaminant solubility and extraction (*8*). In mobilization, surfactants are applied to decrease the IFT of the NAPL-water system. Reductions in IFT can result in displacement of entrapped and pooled DNAPL, and the mobile DNAPL can be recovered as a distinct liquid phase (free product) (*37*). Purchase and ultimate disposal of surfactants when applied at the high concentrations (4–8 wt%) necessary for substantial solubilization and/or mobilization are significant components of SEAR operational costs (*38*).

2.4.1.2 Cosolvent Flushing. Cosolvent (alcohol) flushing is similar to SEAR in objective, mode of action, and field application. Cosolvents do not form micelles but rather increase contaminant dissolved-phase concentrations by making the aqueous phase less polar (*8*). Hydrophobic compounds are generally much more soluble in alcohols (e.g., ethanol) than water; therefore, the equilibrium solubility of sparingly soluble organic contaminants increases with increasing amounts of a water-miscible alcohol (*8*). Alcohols may also reduce IFT, resulting in displacement of DNAPL. The solubility

enhancement or IFT reduction achieved with cosolvents is not as significant as observed with surfactants; thus much higher alcohol concentrations (70–95 wt%) are required to achieve equivalent contaminant recoveries (*8*). Alcohols are substantially less expensive (per unit mass) than surfactants, making cosolvent flushing and SEAR economically comparable (*8*). Significant disadvantages of cosolvent flushing include gravity override (bypassing) of contaminated zones due to density differences between aquifer groundwater and the cosolvent solution, reduced microbial activity as a result of cosolvent toxicity, and safety hazards associated with the flammable and explosive nature of concentrated alcohol solutions (*8,22*).

2.4.2 Chemical Oxidation

Source zone remediation utilizing strong chemical oxidants (e.g., ozone, sodium or potassium permanganate, persulfate, Fenton's reagent) delivered and distributed within DNAPL source zones is known as *in situ* chemical oxidation (ISCO) (*23*). ISCO relies on rapid reaction of the oxidant (or reactive species [e.g., $OH^{\bullet}$] produced by the oxidant) and the dissolved- or NAPL-phase contaminant to produce benign products (i.e., CO_2 and salts) (*8*). Field application of ISCO is relatively effective when treating low levels of dissolved-phase chlorinated solvents and hydrocarbons, but few demonstrations of ISCO for DNAPL treatment have been performed (*8,23*). As outlined in reference (*23*), the factors controlling ISCO performance for DNAPL source zone treatment are: (i) delivery, distribution, and sufficient mixing of chemical oxidants in heterogeneous and low-permeability soils, (ii) overcoming natural oxidant demand, (iii) gas formation and emission, (iv) permeability losses, and (v) potential decreases in water quality (e.g., due

to increased metal mobility or reduced pH (*8,22*)) . Since ISCO may result in a "skin" or "crust" on NAPL surfaces, this technology may also become self-limiting and ultimately increase the mass transfer resistance from the organic to the aqueous phase (*23*).

2.4.3 Thermal Treatment

Thermal treatment technologies, including steam enhanced extraction (SEE), conductive heating, and resistive heating, are designed to deliver thermal energy (heat) to source zones (*8,22,23,39*). SEE consists of direct steam injection into DNAPL source zones. Subsurface groundwater is heated (typically to 100–140ºC) by the applied steam, resulting in volatilization and/or mobilization of the DNAPL. SEE, like many source zone remedies, is most effective in homogeneous and high-permeability soils, although many cheaper alternatives exist for application to high-permeability soils. DNAPL mobilization and redistribution to deeper or previously uncontaminated areas of the aquifer may also occur during SEE. In conductive heating, the subsurface temperature (100–500ºC) is increased by heat conduction from electrical heating elements, which can reach temperatures as high as 900ºC. Such heating results in contaminant vaporization. Subsurface temperatures during conductive heating are relatively uniform, regardless of subsurface heterogeneity. Electric resistive heating (ERH) employs electrode arrays to provide thermal energy to contaminanted aquifer materials. ERH can increase the subsurface temperature to above the boiling point of water, creating steam *in situ* that strips contaminants from the subsurface. Typically, a more uniform distribution of temperatures occurs in ERH than in SEE or conductive heating. The major advantage of ERH is that this technology works in low permeability systems. Contaminant recovery

occurs via liquid and/or vapor extraction from the subsurface during the thermal treatments. Alternatively, some contaminant mass may also be removed by oxidation or pyrolysis during thermal treatment (*39*). The main limitations to thermal treatment are the potential for NAPL mobilization into previously uncontaminated areas, potential formation of undesirable intermediates or degradation products, and the high energy costs associated with the technology (*8,22,23*).

2.4.4 Benefits and Limitations of Physical-Chemical Source Zone Remediation

Physical-chemical source zone remediation technologies, including surfactant enhanced aquifer remediation (SEAR), cosolvent flushing, chemical oxidation, and thermal treatment, can remove or destroy significant amounts (50–90%) of contaminant mass relatively quickly, especially when compared to traditional pump-and-treat approaches (*8*). Such reductions in source zone longevity may decrease the length and severity of environmental and health risks (*23*). Additional benefits of source zone depletion technologies (e.g., reductions in DNAPL mobility, decrease in mass discharge and plume formation, enhanced efficiency of complementary technologies) are extensively discussed in reference (*23*). Although successes have been documented with currently-available physical-chemical remedies in both laboratory studies and field trials, there is considerable uncertainty in the benefits of such treatments (Stroo et al. (*10*) provides an excellent review). In fact, these aggressive techniques rarely reach typical cleanup levels, and near-term environmental risks may not be significantly reduced since post-treatment contaminant concentrations emanating from treated DNAPL source zones can still exceed regulatory limits (*10,40*). Physical-chemical remediation technologies

are expensive in the short term, and it is often hard to predict or guarantee the long-term effectiveness and economic return for such initial investments (*8,10,23*). In fact, even the metrics (e.g., mass removal, decreases in dissolved-phase concentrations, decreases in mass flux, economic considerations) used to evaluate the success of physical-chemical remedies are currently debated within the remediation and regulatory community (*8,10,23*). As discussed above, these remedies may result in detrimental changes in the distribution and/or the physical and chemical characteristics of remaining DNAPL, potentially making future remediation more challenging (*8,10,23*). Although current research is addressing the potential limitations of physical-chemical source zone remediation and the benefits of partial mass removal from DNAPL source zones (e.g., see reference (*27*)), it is likely that alternative approaches (e.g., passive remedial approaches like bioremediation) or tertiary ("polishing") strategies need to be implemented to replace or follow physical-chemical treatments (*8,22,23,41*). Source zone bioremediation and the use of complementary, coupled technologies are discussed in Sections 2.6 and 2.7, respectively.

2.5 Biological Transformation of Chlorinated Ethenes

Bioremediation exploits the catalytic potential of microorganisms to degrade and/or detoxify contaminants. Demonstration of bioremediation as a viable technology first began with petroleum hydrocarbon contamination of surface soils in the 1970s and 80s (*7*). Over the last decade, bioremediation has gained increased acceptance as an *in situ* technology for subsurface and groundwater contaminants (e.g., chlorinated ethenes)

(*7*). Chlorinated ethenes can be detoxified by a variety of microorganisms in both oxidative and reductive processes (*42*). Table 2.2 provides an overview of the microbial processes involved in the transformation of chlorinated ethenes; for a thorough discussion and review of these processes, see references (*5,42-47*). Although potentially relevant as viable bioremediation or natural attenuation strategies for lesser chlorinated ethenes (*cis*-DCE and VC), oxidative processes are not currently applicable to PCE or TCE (see Table 2.2) (*22*). Sequential coupling of anaerobic and aerobic processes might lead to complete PCE detoxification (*18,48*). Cometabolic processes are slow, often incomplete, and depend on the presence of a primary substrate (e.g., methane, toluene) (*22*). Such fortuitous reactions, therefore, only allow for indirect control over the process of interest. Due to the current shortcomings of oxidative and cometabolic processes and the prevalence of anaerobic conditions at many contaminated sites, microbial reductive dechlorination presently holds the most promise for remediation of chlorinated ethenes (*7*).

2.5.1 Microbial Reductive Dechlorination of Chlorinated Ethenes

During metabolic microbial reductive dechlorination (i.e., [de]chlororespiration), chlorinated organic compounds serve as terminal electron acceptors in a respiratory (metabolic) process of specialized bacteria, resulting in energy conservation and ultimately bacterial growth (*49*). Reductive dechlorination couples contaminant transformation to the oxidation of suitable substrates (i.e., electron donors) (*49*). PCE and TCE can be reductively dechlorinated under anaerobic conditions to sequentially form less-chlorinated daughter products, as shown in Figure 2.4. At each step, a chlorine

Table 2.2 Summary of aerobic and anaerobic microbial processes involved in transformation of chlorinated ethenes [a].

	Oxidation Processes		Cometabolic Processes		Microbial Reductive Dechlorination
	Anaerobic	**Aerobic**	**Anaerobic Reduction**	**Aerobic Oxidation**	
Metabolic group(s)	Fe(III) reducers Mn(IV) reducers Humic acid reducers	*Mycobacterium* spp. *Nocardioides* spp. *Pseudomonas* spp. *Polaromonas* sp.	Sulfidogens Methanogens Acetogens	Organisms with broad range oxygenases	Chlororespirers (see Section 2.5.1)
Energy yielding	Yes	Yes	No	No	Yes
Chlorinate ethenes transformed	*cis*-DCE, VC	*cis*-DCE, VC	PCE, TCE	PCE, TCE, *cis*-DCE, VC	PCE, TCE, *cis*-DCE, VC
Frequency of active organisms in nature	Unknown	VC oxidizers widely distributed in aerobic environments	High in anaerobic environments	High in aerobic environments	Not rare in anaerobic environments
Favorable site conditions	Fe(III) reducing Mn(IV) reducing	Aerobic	Anaerobic, not e^- donor or e^- acceptor limited	Aerobic, primary substrate present	Anaerobic, appropriate e^- donor present, no interfering TEAPs
Comments	Poorly understood; relevance unclear	Not currently applicable to PCE and TCE; sequential coupling with reductive dechlorination might lead to detoxification	Low rates; incomplete dechlorination	Indirect control over process of interest; dechlorination often stalls due to accumulation of toxic intermediates	Incomplete dechlorination sometimes observed

[a] Adapted from Christ et al. (*22*); abbreviations: e^-, electron; TEAPs, terminal electron-accepting processes

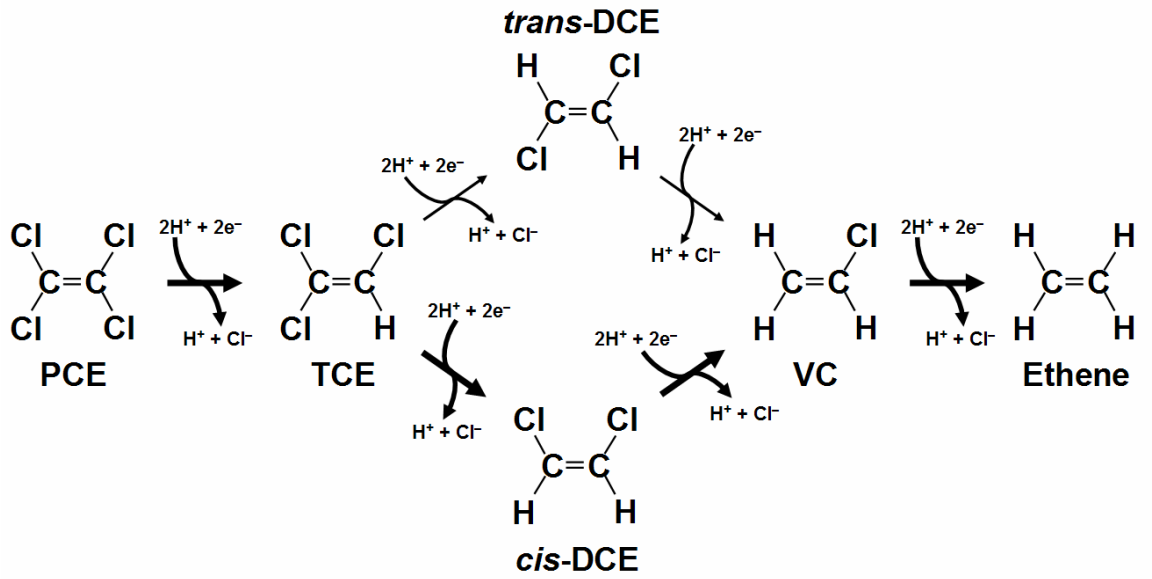

Figure 2.4 Microbial reductive dechlorination of PCE to ethene. The thick arrows indicate the predominate pathway (adapted from Löffler and Edwards (*7*)).

substituent is replaced by hydrogen, releasing a chloride ion and a proton (an acidifying process). Complete dechlorination of PCE to ethene results in contaminant detoxification, since ethene is benign. As discussed above, accumulation of intermediates (e.g., VC) is undesirable since the daughter products also pose environmental and health risks.

Over the last two decades, considerable effort has been devoted to identifying and isolating bacteria capable of metabolic reductive dechlorination (*49*). As a result, many dechlorinating organisms have been identified from both contaminated and uncontaminated (pristine) environments (*49*). Dechlorinating isolates are found in phylogenetically diverse bacterial groups, including both Gram-positive and Gram-negative bacteria. Several microbial isolates only transform PCE to TCE (e.g., *Desulfitobacterium* sp. strain Viet1 (*50,51*)) or *cis*-DCE (e.g., *Desulfuromonas* spp. (*52,53*), *Sulfurospirillum* spp. (*54,55*), *Geobacter lovleyi* strain SZ (*56*), *Desulfitobacterium* spp. (*57,58*), and *Dehalobacter* spp. (*59,60*)). A partial list of PCE-to-TCE and PCE-to-*cis*-DCE dechlorinators and their electron donors is provided in Table 2.3.

A milestone for the success of chlorinated ethene bioremediation was the identification of *Dehalococcoides* spp. as the catalysts involved in reductive dechlorination of DCEs and VC to innocuous products (i.e., ethene and inorganic chloride) (*61-70*). Currently, only members of the *Dehalococcoides* group have been shown to possess this catalytic ability. As shown in Table 2.4, none of the available *Dehalococcoides* isolates grow with all chlorinated ethenes as electron acceptors, and some transformation steps are slow and cometabolic (i.e., not linked to energy capture

Table 2.3 Select reductively dechlorinating isolates capable of incomplete dechlorination of PCE (adapted and updated from Löffler et al. (*49*)).

Lineage	Organism	Dechlorination Activity	Electron Donors [a]	Reference
Low G+C Gram-positive bacteria	*Desulfitobacterium hafniense* strain Y51	PCE to *cis*-DCE	H_2	(*58*)
	Desulfitobacterium sp. strain Viet1	PCE to TCE	H_2	(*50,51*)
	Dehalobacter restrictus strain PER-K23	PCE to *cis*-DCE	H_2	(*59,60*)
	Culture "*Clostridium bifermentans* strain DPH1"	PCE to *cis*-DCE	H_2	(*71*)
Proteobacteria, δ-subdivision	*Desulfuromonas chloroethenica* strain TT4B	PCE to *cis*-DCE	acetate	(*52,72*)
	Desulfuromonas michiganensis strain BB1	PCE to *cis*-DCE	acetate	(*53*)
	Desulfuromonas michiganensis strain BRS1	PCE to *cis*-DCE	acetate	(*53*)
	Geobacter lovleyi strain SZ	PCE to *cis*-DCE	H_2, acetate	(*56*)
Proteobacteria, ε-subdivision	*Sulfurospirillum multivorans* (formally *Dehalospirillum multivorans*)	PCE to *cis*-DCE	H_2	(*55,73*)
	Sulfurospirillum halorespirans	PCE to *cis*-DCE	H_2	(*54*)
Proteobacteria, γ-subdivision	*Enterobacter agglomerans* strain MS-1 [b]	PCE to *cis*-DCE	acetate [c]	(*74*)

[a] Indicates the ability to use H_2 or acetate as electron donor for reductive dechlorination. Most species are more versatile and use a variety of electron donors. Of the listed species, only *Dehalobacter* spp. are strict hydrogenotrophic populations.

[b] Growth linked to reductive dechlorination has not been unequivocally demonstrated.

[c] H_2 was not tested.

Table 2.4 Properties of select *Dehalococcoides* strains involved in chlorinated ethene reductive dechlorination (adapted from Löffler and Edwards (*7*)) [a].

***Dehalococcoides* strain**	**Metabolic chlorinated ethene electron acceptors**	**Cometabolized chlorinated ethenes**	**Major end products**	**Biomarker RDase**	**Reference**
Strain 195	PCE, TCE, *cis*-DCE, 1,1-DCE	*trans*-DCE, VC	VC, ethene	*tceA*	(*67,68,75*)
Strain FL2	TCE, *cis*-DCE, *trans*-DCE	PCE, VC	VC, ethene	*tceA*	(*65*)
Strain BAV1	*cis*-DCE, *trans*-DCE, 1,1-DCE, VC	PCE, TCE	Ethene	*bvcA*	(*64,76*)
Strain VS [b]	TCE, *cis*-DCE, 1,1-DCE, VC	ND	Ethene	*vcrA*	(*61,62,69*)
Strain GT	TCE, *cis*-DCE, 1,1-DCE, VC	None	Ethene	*vcrA*	(*70*)

[a] Abbreviations: RDase, reductive dehalogenase, ND, not determined

[b] Characterized in a mixed culture

and growth). Other *Dehalococcoides* strains cannot dechlorinate chlorinated ethenes but dechlorinate other chlorinated contaminants (e.g., chloropropanes (*77*)). Therefore, the detection of *Dehalococcoides* organisms does not guarantee efficient ethene formation (*7*). Phylogenetically, *Dehalococcoides* are deeply branching green non-sulfur bacteria (*Chloroflexi*) with an unusual cell wall structure and membrane composition (*68,78*). *Dehalococcoides* are strictly hydrogenotrophic (de)chlororespirers (i.e., they require chlorinated organic compounds as electron acceptors and hydrogen as electron donor), and genetic and genomic information from *Dehalococcoides* organisms suggest that they are capable of dechlorinating a wide variety of chloroorganic substrates (*7*). *Dehalococcoides* isolates are fastidious organisms that prove difficult to grow and maintain in pure culture (*7*). The reason(s) for the intricate growth of *Dehalococcoides* spp. in pure culture are unclear but may be due to unknown nutritional requirements and/or sensitivity to redox conditions and oxygen (*64,68*). *Dehalococcoides* organisms, therefore, are more easily maintained as part of dechlorinating consortia (i.e., mixed cultures) (*7*).

Transformation of chlorinated ethenes during microbial reductive dechlorination is catalyzed by a novel class of redox enzymes, reductive dehalogenases (RDases) (*49*). RDase enzyme systems have been purified and characterized from a few PCE-to-*cis*-DCE dechlorinating isolates (as reviewed in reference (*49*)). In addition, several RDase genes implicated in chlorinated ethene dechlorination have been identified in *Dehalococcoides* organisms (e.g., see Table 2.4) (*76,79-82*). A TCE-to-VC RDase (TceA) was identified and characterized from *Dehalococcoides ethenogenes* strain 195 (*75*). The gene encoding the TCE RDase, *tceA*, was also identified in *Dehalococcoides*

sp. strain FL2 (*65*). A *cis*-DCE-to-ethene RDase (VcrA) was purified from strain VS (*69*), and the gene encoding for this RDase (*vcrA*) was also identified in strain GT (*70*). A putative VC-to-ethene RDase (BvcA) was identified in strain BAV1, and the gene encoding this RDase (*bvcA*) was detected in several VC-respiring mixed cultures (*76*). As discussed in Section 2.8, identification of RDase genes (i.e., *tceA*, *bvcA*, and *vcrA*) associated with chlorinated ethene transformation processes has allowed for the design of RDase gene-targeted molecular tools specific to critical dechlorination steps. Identification of these process-specific indicator genes (biomarkers) allows for a more accurate characterization of the *Dehalococcoides* population (*83,84*).

2.5.2 Biostimulation and Bioaugmentation for Aquifer Remediation

Bioremediation (i.e., biostimulation and bioaugmentation) utilizing the microbial reductive dechlorination process has been implemented in the field to contain and treat chlorinated ethene plumes (e.g., see references (*85-88*)). Due to recent successes, bioremediation is becoming increasingly accepted as a viable remediation strategy (*89*). Dechlorinating organisms are native to many sites contaminated with chlorinated ethenes, but the dechlorinating bacteria are often patchily distributed throughout the aquifer and/or are present in very low numbers (*85,86*). At sites where native dechlorinators occur, lack of substrates (i.e., electron donor) and/or unfavorable geochemical conditions (e.g., pH, redox) often limit (i.e., control) reductive dechlorination and contaminant detoxification. To overcome the nutritional bottleneck(s) in oligotrophic aquifers, biostimulation with organic (e.g., lactate) and inorganic (i.e., N and P) substrates has been successfully implemented at the field scale to promote complete dechlorination of PCE to ethene (*85*).

Many organic substrates have been proposed and employed as biostimulatory agents, including alcohols, organic acids, emulsified vegetable oil, and complex organic materials (e.g., molasses, corn cobs, chitin) (*7,89*). Fermentation of these compounds induces anaerobiosis (favorable redox conditions) and supplies suitable electron donors (i.e., H_2, acetate). Although biostimulation has been employed productively at several sites, this approach may be insufficient to sustain desirable dechlorination rates and only works at sites that have native dechlorinators (i.e., *Dehalococcoides* spp.) capable of efficient PCE dechlorination to ethene. As an alternative approach, robust PCE-to-ethene dechlorinating consortia are grown *ex situ*, transported to the contaminanted site, and injected into the subsurface in a process called bioaugmentation. This approach, which is usually accompanied by biostimulation, has been implemented at numerous sites (*85-88*), and bioaugmentation inocula are commercially available (e.g., Bio-Dechlor INOCULUM [Regenesis, http://www.regenesis.com]; KB-1 [SiREM, http://www.siremlab.com]; SDC-9 [Shaw, http://www.shawgrp.com]). State-of-the-art bioaugmentation application includes techniques that reduce exposure of the bioaugmentation culture to air (*90*); however, for practical reasons this is difficult to achieve at many contaminanted sites and may limit the ultimate performance of the bioaugmented, strictly anaerobic organisms *in situ*. Another challenge facing successful bioaugmentation is distributing the dechlorinating bacteria throughout the targeted remedial zone (*90*). Adhesion, attachment, and colonization of bacteria in subsurface environments are poorly understood (*89,90*); these factors may restrict bioaugmentation approaches under certain environmental conditions. For further information about the development, current status, and avenues of future research for bioaugmentation technologies, see reference (*90*).

2.6 Source Zone Bioremediation

Although both biostimulation and bioaugmentation have been successfully implemented in the field to promote reductive dechlorination of dissolved-phase contaminant and to contain chlorinated ethene plumes, these approaches do not reduce overall remediation time since the rate of contaminant dissolution from the DNAPL source zone remains unchanged. Therefore, just as with physical-chemical technologies, the focus of research and development of bioremediation technologies has changed from plume containment to treatment of DNAPL source zones (*8*). The feasibility of this approach is thought to depend on the activity of dechlorinating microorganisms at high, saturated PCE concentrations expected near the NAPL-water interface and frequently encountered in PCE-DNAPL source zones (*23*). Source zone bioremediation once seemed infeasible since free-phase PCE and high PCE concentrations were toxic and/or inhibitory to many dechlorinating microorganisms (for an extensive summary of the tolerance of chlororespiring isolates and enrichment cultures to elevated PCE concentrations, see Chapter 3, Table 3.1). Recently, dechlorinating isolates and mixed consortia were reported to dechlorinate PCE at or near saturated concentrations (see Chapter 3, Table 3.1), suggesting that biologically enhanced (bioenhanced) DNAPL dissolution is possible.

There are several potential mechanisms by which biological activity can enhance PCE-DNAPL dissolution. Currently, the prevailing wisdom is that biological activity in proximity of the NAPL-water interface increases the driving force for PCE dissolution as a result of reduced PCE concentrations in the bulk, aqueous phase (C_a^{PCE}; see Equation

2.1 and Figure 2.2). That is, microbial activity increases the difference between the PCE concentration in the aqueous phase at equilibrium with the organic phase ($C_{ao}^{PCE\text{-}e}$) and the concentration in the bulk, aqueous phase (C_a^{PCE}), resulting in greater contaminant dissolution. Alternative mechanisms of bioenhanced dissolution include decreasing the boundary layer thickness (δ; see Figure 2.2) or direct attachment of dechlorinating organisms on the surface of the NAPL (see Figure 2.5). Further studies are needed to elucidate microbial interactions at the NAPL-water interface that result in bioenhanced dissolution (*8,10*). Biological activity in the proximity of DNAPL can also affect interphase mass transfer by transforming PCE to compounds that are less hydrophobic and more soluble (e.g., DCEs) (*91,92*).

Several experiments in continuous-flow stirred-tank reactors, one-dimensional (1-D) columns, two-dimensional (2-D) aquifer cells, and three-dimensional (3-D) simulated aquifers have been conducted to evaluate microbial reductive dechlorination performance and bioenhanced dissolution in simulated PCE-DNAPL source zones (see Table 2.5) (*21,91-97*). These experiments demonstrated that the microbial reductive dechlorination process can result in bioenhanced (1.3 – 14 times) DNAPL dissolution. Although these findings are promising, significant accumulation of toxic intermediates and only minimal amounts of benign ethene were observed in these studies (see Table 2.5). Several reasons for incomplete PCE dechlorination have been proposed: insufficient contact/residence times (*16,96*), reduced system pH (*92,98*), inhibitory levels of polychlorinated ethenes (PCE, TCE, and *cis*-DCE) (*96,99,100*), and inadequate supply of electron donor due to bioclogging (*95,96,101*).

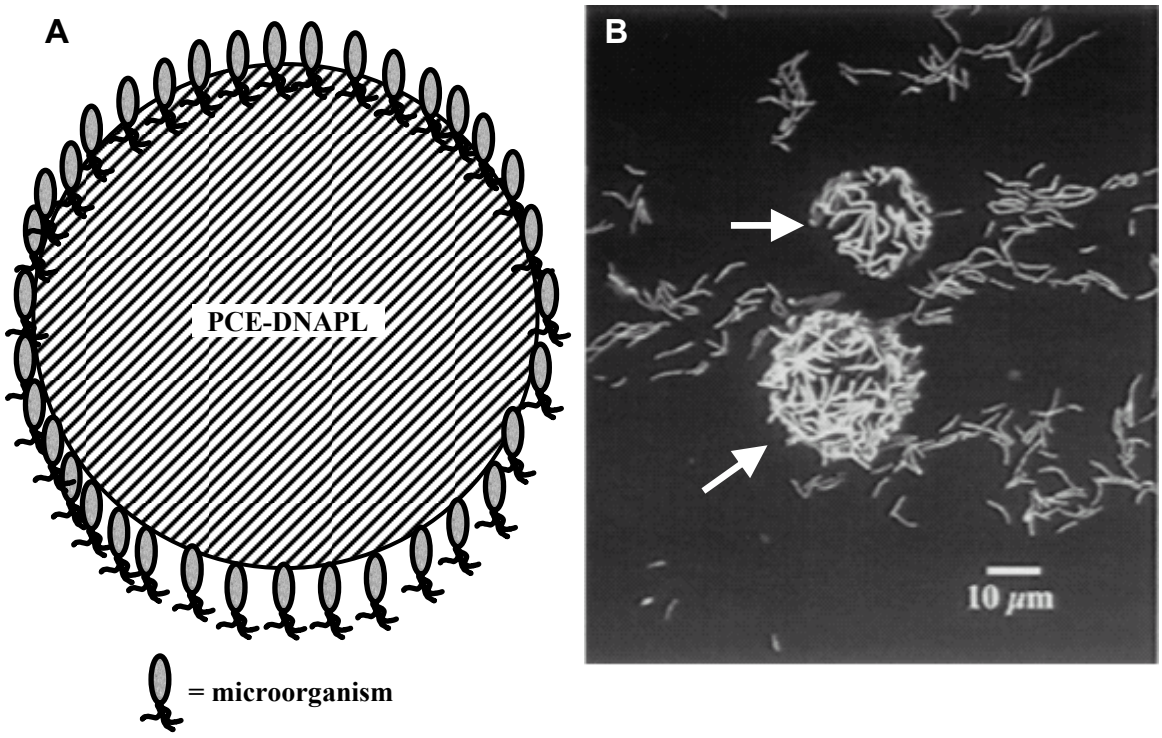

Figure 2.5 Microbial attachment to NAPL surfaces. (A) Simplified representation of direct attachment of dechlorinating organisms to the surface of PCE-DNAPL, a possible mechanism by which microbial activity enhances DNAPL dissolution. (B) Cells of *Rhodococcus* sp. strain Q15 covering the surface of a light nonaqueous phase liquid (diesel fuel microdroplets; see arrows), suggesting that this organism assimilates liquid alkane substrates by adhering to the alkane phase. Panel (B) was adapted and modified from Whyte et al. (*102*).

Table 2.5 Summary of select bioenhanced PCE-NAPL dissolution experiments (adapted and updated from the *Supporting Information* of Sleep et al. (*94*)).

Reference Study	System Description	Major Dechlorination Product(s) [a]	Dissolution Enhancement
Yang and McCarty, 2000 (*21*)	1-D column containing aquifer material and PCE-DNAPL	*cis*-DCE	5
Carr et al., 2000 (*91*)	Continuous-flow stirred-tank reactor with PCE/tridecane NAPL (0.13 mol/mol PCE/tridecane) [b]	*cis*-DCE	14
Cope and Hughes, 2002 (*92*)	1-D column containing glass beads with PCE/tridecane NAPL (0.13 mol/mol PCE/tridecane) [b]	TCE, VC	1.3 – 6.5
Yang and McCarty, 2002 (*95*)	1-D column containing aquifer material and PCE-DNAPL	*cis*-DCE	2.1 – 3
Adamson et al., 2003 (*93*)	3-D pilot-scale simulated aquifer containing sand and PCE-DNAPL	*cis*-DCE, VC	Not determined
Sleep et al., 2006 (*94*)	2-D aquifer cell containing aquifer material and PCE-DNAPL	*cis*-DCE, VC	1.7 – 3.3
Da Silva et al., 2006 (*96*)	3-D pilot-scale simulated aquifer containing sand and PCE-DNAPL	*cis*-DCE, VC	1.6
Glover et al., 2007 (*97*)	Custom 5-cm flow cell containing sand mixtures (course- to fine-grained) and PCE-DNAPL	*cis*-DCE	<1.5 [c] 4-13 [c]

[a] All experiments were performed with dechlorinating consortia capable of transforming PCE to VC and ethene.

[b] The NAPL had a density ~0.81 g per mL and therefore was not a DNAPL

[c] Enhancement depended on average DNAPL pool saturations (<0.55, enhancement of 4-13; 0.74, enhancement of <1.5) (*97*)

Although the previous bioenhanced dissolution studies indicate dechlorination activity in the vicinity of PCE-DNAPL and suggest microbial colonization of the source zone by dechlorinating organisms, few of these studies evaluated microbial community dynamics and composition during bioenhanced dissolution. Adamson et al. (*93*) used denaturing gradient gel electrophoresis (DGGE) and polymerase chain reaction (PCR) to evaluate changes in microbial diversity and the presence of *Dehalococcoides* spp. respectively, in a limited number of samples from their near-field scale simulated aquifer. DGGE indicated reduced microbial diversity in the simulated aquifer as compared to the bioaugmentation inoculum, while *Dehalococcoides* spp. were detected in the two samples from the simulated aquifer. Sleep et al. (*94*) also used DGGE as well as quantitative real-time PCR (qPCR) to gather microbiological information during bioenhanced dissolution. After bioaugmentation of the 2-D aquifer cell, the number of *Dehalococcoides* cells detected in the effluent increased by approximately three orders-of-magnitude, corresponding to a significant decrease in PCE concentrations and an equivalent increase in PCE dechlorination products (mainly *cis*-DCE). Interestingly, the highest number of *Dehalococcoides* organisms (10^7 cells per gram of soil) was detected in soil samples taken nearest the DNAPL source zone. Although microbial growth was observed throughout the aquifer cell, spatial heterogeneity of microbial species was observed. Given the limited number of sampling locations (5 sampling ports in the 76 cm x 38 cm x 2.54 cm aquifer cell), the spatial heterogeneity could not be well characterized, especially in relationship to the PCE-DNAPL source zone.

2.7 Complementary Technologies

As described above, there are limitations to physical-chemical as well as biological remediation strategies for successful source zone treatment. Recent attention, therefore, has focused on complementary technologies that can be applied together or sequentially to reduce contaminant mass flux, control long-term plume development, and achieve remedial goals (*22,23,41,103-105*). In a coupled "treatment train" scenario, a physical-chemical remedy removes significant contaminant mass and another tertiary ("polishing") technology is applied to effectively remove or detoxify the remaining contaminant (*22*). Sequential approaches have the potential to overcome the challenges associated with each technology when used in isolation (*22,41,103*). The impacts of aggressive technologies on the effectiveness of polishing remedies are insufficiently explored (*10*). For example, physical-chemical treatments may alter geochemical and microbiological conditions that may be detrimental (e.g., aquifer clogging, reduced microbial diversity) or beneficial (e.g., enhanced electron donor availability) to polishing technologies (*10,22*). A commonly considered polishing technology is microbial reductive dechlorination (*22,23,41,103*), and combinations of SEAR, cosolvent flushing, ISCO, or thermal treatment with subsequent microbial activity have been proposed (*10*). Recent studies have evaluated the impact of physical-chemical remedies on subsequent biological activity (SEAR (*41,106,107*); cosolvent flushing (*103,104*); ISCO (*108,109*); and thermal treatment (*105,110-114*)), but the results, although promising, are inconclusive. Existing experimental and field evidence suggests that SEAR and cosolvent flushing followed by microbial reductive dechlorination are the most promising

avenues for application of combined remedies (*22*). Section 2.7.1 discusses the advantages and current shortcomings of coupling SEAR and bioremediation; many of the discussed topics are also germane to sequential application of cosolvent flushing and microbial reductive dechlorination.

2.7.1 SEAR Coupled with Microbial Reductive Dechlorination

There are many potential benefits of coupling SEAR with microbial reductive dechlorination. Application of SEAR can reduce dissolved-phase contaminant concentrations within and emanating from source zones (*27*), possibly to levels that are more amenable to subsequent microbial reductive dechlorination activity. Although recovery of surfactant is generally high for well designed SEAR systems (e.g., up to 95% (*115,116*)), residual surfactant will remain in the aquifer. The remaining surfactant may actually be beneficial for subsequent microbial reductive dechlorination activity. For example, degradation of the surfactant may consume oxygen and promote anaerobiosis. Such conditions are prerequisite for microbial reductive dechlorination and are likely to occur, since many surfactants are readily degraded under aerobic and anaerobic conditions (*117-119*). Surfactant degradation may also produce hydrogen and organic acids (e.g., acetate) that can provide reducing equivalents to support microbial reductive dechlorination (*41*). Alternatively, residual surfactant may increase contaminant bioavailability, thereby enhancing microbial transformation (*120*).

Stimulation of microbial reductive dechlorination activity was recently observed at the Bachman Road site in Oscoda, Michigan, following SEAR (*41*). At the Bachman Road site, a pilot-scale field demonstration of SEAR was performed using a 60,000 mg/L

solution of Tween 80 (a nonionic, food-grade surfactant; see Figure 2.3) to solubilize and remove PCE-DNAPL from the source zone (*41,115,116*). In the Bachman aquifer, elevated concentrations (>2 orders-of-magnitude greater than pretreatment values) of PCE dechlorination products (e.g., *cis*-DCE), as well as elevated levels of organic acids (e.g., acetate), were observed in monitoring wells 450 days following the cessation of surfactant flushing (*41*). Fermentation of residual Tween 80 (detected at 50 – 2,750 mg/L 450 days after SEAR) provided suitable electron donors(s) that stimulated native microbial dechlorination activity in the oligotrophic aquifer (*41*).

For microbial reductive dechlorination to be considered feasible following SEAR, the selected surfactant(s) should have little or reversible impact (i.e., toxicity, inhibition) on the microbial populations relevant for the detoxification process. McGuire and Hughes (*106*) reported the effect of several nonionic, anionic, and cationic surfactants on the dechlorination performance of a PCE dechlorinating consortium. Tween 80 (1,000 – 10,000 mg/L) was the least inhibitory of the tested surfactants, although this surfactant did reduce the rate of ethene formation. Ramsburg et al. (*41*) found that Tween 80 (1,000 – 5,000 mg/L) did not alter the dechlorination performance of *Desulfuromonas michiganensis* strain BB1, a PCE-to-*cis*-DCE dechlorinator indistinguishable from dechlorinating strain BRS1 present at the Bachman Road site (*53*). Yeh et al. (*121*) observed that Tween 80 inhibition of hexachlorobenzene reductive dechlorination by three mixed cultures was dependent on surfactant concentration, with no inhibition observed at 10 mg/L, decreased dechlorination rates at 200 mg/L, and complete inhibition of dechlorination at 1,000 mg/L. These observations suggest that Tween 80 effects on dechlorinators are complex and poorly understood. Additional research, therefore, is

needed to explore the possible stimulatory and inhibitory effects of surfactants (at concentrations similar to the residual levels following SEAR) on microbial reductive dechlorination (*22*). Such understanding is vital for successful application of sequential remedies to a broad range of contaminanted sites.

2.8 Molecular Biological Tools

The application of cultivation-independent molecular biological tools (MBTs) to assess contaminanted sites and monitor bioremediation has greatly improved the ability of scientists and engineers to implement biological remedies and to establish cause-and-effect relationships between microbial processes and contaminant detoxification (*7,122*). Use of these diagnostic tools, therefore, has gained increased acceptance with both regulators and environmental practitioners (*7,122*). This section presents a brief summary of select nucleic acid-based MBTs that have proven useful in obtaining qualitative and quantitative microbiological information during laboratory experiments, site assessment, and bioremediation performance monitoring. Further information about the application of MBTs in support of environmental remediation can be found in reference (*122*).

2.8.1 Nucleic Acid Biomarkers

Nucleic acid-based MBTs allow for specific and sometimes quantitative detection of key bacteria involved in detoxification processes (*7*). Currently, nucleic acid-based MBTs are frequently used to detect and/or quantify DNA biomarkers (i.e., genes) (*123*).

The most common target for nucleic acid-based MBTs is the bacterial 16S rRNA gene (*122*). In general, the 16S rRNA gene provides relevant information about an organism's phylogeny and often physiology. The 16S rRNA gene is targeted since (i) it is present in all bacteria, (ii) an extensive 16S rRNA gene sequence database exists that aids in design of specific 16S rRNA gene-targeted MBTs, and (iii) changes (evolution) in this gene occur slowly due to the critical role that 16S rRNA plays in the function of ribosomes. 16S rRNA gene-targeted MBTs are typically used for detection of a particular organism or group(s) of organisms known to be key players in a specific process (*122*). Certain processes are carried out by phylogenetically-distinct bacterial groups or only certain members of a phylogenetically-coherent group (*84,122*). For such processes, phylogenetic information obtained from 16S rRNA gene analysis may not be useful to predict metabolic function (phenotype) (*122*). When 16S rRNA gene-targeted MBTs fail to provide sufficient resolution for specific detection of organism(s) involved in processes of interest, identification of alternative targets indicative of desired metabolic processes (e.g., "functional" genes that code for proteins involved in contaminant transformation) are needed. For example, certain members of the *Dehalococcoides* group have highly similar or even identical 16S rRNA genes but diverse metabolic capabilities (*7,84,122*). Identification of reductive dehalogenase (RDase) genes (i.e., *tceA*, *bvcA*, and *vcrA*) associated with specific chlorinated ethene transformation reactions (e.g., (*69,75,76*)) has allowed for design of RDase gene-targeted MBTs specific to critical dechlorination steps (*83,84*). Use of both phylogenetic (16S rRNA gene-based) and process-specific (functional gene-based) MBTs are important for understanding microbial processes involved in contaminant detoxification and have further enhanced the ability of

site managers to effectively implement reliable, cost-effective bioremediation approaches (*7,85,86,122*).

Although DNA biomarkers are useful for indicating metabolic potential (i.e., the presence of specific bacteria or functional genes), detection of DNA biomarkers does not always indicate that the desired metabolic activity is occurring or that the target populations are active (*122*). Unlike DNA biomarkers, detection of RNA biomarkers (i.e., gene transcripts) is considered to be a more direct measure of microbial activity (*7,122*). In RNA biomarker analysis, functional gene transcription is monitored by detecting or enumerating mRNA transcripts. Typically, mRNA is extracted from sample material and then converted to cDNA by reverse transcription (RT) (e.g., see reference (*76*)). Application of MBTs with cDNA then proceeds as with DNA. The lack of established protocols for RNA biomarker analysis and concerns related to RNA biomarker stability make interpreting and comparing results from RNA biomarker analysis difficult (*122*). Detailed studies are needed to address the current limitations of RNA biomarker analysis and to increase the resolution and applicability of this approach. Despite the current limitations, RNA-targeted MBTs have already proven useful in laboratory experiments (*76,82,124-127*), and current studies are evaluating the applicability of RNA-targeted MBTs for field assessment and determining *in situ* microbial activity (*128,129*).

2.8.2 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a powerful tool in environmental forensics. For an excellent web-based demonstration of the fundamentals of PCR, please see

http://www.dnalc.org/ddnalc/resources/pcr.html (Dolan DNA Learning Center, Cold Spring Harbor Laboratory, New York). In PCR, specific nucleotide sequences are amplified, resulting in millions of copies of the targeted sequence and making detection of small numbers of DNA or RNA biomarkers possible (*123*). PCR protocols have been designed that specifically amplify the 16S rRNA gene of many dechlorinating organisms, including *Dehalococcoides* spp. (*63,66,123*), *Desulfuromonas* spp. (*123*), *Sulfurospirillum* spp. (*130,131*), *Desulfitobacterium* spp. (*132*), and *Dehalobacter* spp. (*133*). PCR-based tools have not been developed for specific and sensitive detection of *Geobacter lovleyi* strain SZ. Additional protocols have been designed to specifically target RDase genes, including *pceA* of *Sulfurospirillum multivorans (134)*, *pceA* of *Dehalobacter restrictu*s (*134*), *tceA* of *Dehalococcoides ethenogenes* strain 195 (*75,134,135*), *bvcA* of *Dehalococcoides* sp. strain BAV1 (*76*), and *vcrA* of *Dehalococcoides* sp. stains GT and VS (*69*). Direct application of PCR (direct PCR) with DNA extracted from bacterial cultures, groundwater samples, or aquifer solids yields information regarding the presence or apparent absence of a particular organism or biomarker gene (*84,123*). The sensitivity of PCR can be improved by performing nested PCR in which an initial round of amplification occurs using *Bacteria*-targeted or group-specific primers followed by another round of PCR amplification with specific, internal primers (*123,134*). Nested PCR is orders of magnitude more sensitive than direct PCR (see reference (*123*) and the references cited therein). Despite the sensitivity of both direct and nested PCR, these approaches are "endpoint" assays that only provide qualitative (plus/minus) information (*84*).

2.8.3 Quantitative Real-Time PCR (qPCR) Analysis

Direct and nested PCR provide valuable information regarding the presence, distribution, and fate of specific biomarkers. Quantitative real-time PCR (qPCR) analysis provides an additional level of precision by enumerating target biomarkers (*84,122*). qPCR analysis is similar to PCR, but the reaction components also include a fluorescent reporter (e.g., fluorescent dye, fluorophore). During the PCR, fluorescence increases during each PCR cycle, and the amount of fluorescence can be correlated to the number of initial target genes present in the PCR reaction. Many detection chemistries (e.g., TaqMan, SYBR Green) are available for qPCR analysis; for more information about different detection chemistries, please see *Tools and Technologies for Real Time PCR* (Bio-Rad, http://www.biocompare.com/pcr/tutorial/qpcr/flash_go.html). qPCR protocols have been designed for specific detection and quantification of many dechlorinating organisms, including *Dehalococcoides* spp. (*64,83,84,136,137*), *Dehalobacter* spp. (*136*), *Desulfitobacterium* spp. (*136*), and *Geobacter* spp. (*138*). Specific quantitative tools have not been developed for detection of *Sulfurospirillum multivorans* and *Geobacter lovleyi* strain SZ. Protocols have also been designed to quantify RDase genes, including *tceA* (*124,137*), *bvcA* (*83,84*), and *vcrA* (*83,84*). qPCR analysis has a dynamic range spanning 6 to 8 orders-of-magnitude and is generally as sensitive as direct PCR and sometimes nested PCR (e.g., see references (*84,123*)). qPCR analysis was first developed to detect and enumerate DNA biomarkers (i.e., gene copies) but has recently been extended to quantify RNA biomarkers (i.e., transcript copies) via RT-qPCR analysis (*82,124-127*).

When using a SYBR Green-based detection chemistry, melting curve analysis can also accompany qPCR analysis (*139*). Melting (i.e., disassociation) temperatures of double stranded PCR amplicons depend mainly on amplicon size and GC content and can be used as an additional diagnostic tool to verify target sequence amplification (*139-141*). Melting curve analysis provides additional resolution to help distinguish closely related strains, as has been described for medically relevant microorganisms (summarized by Robinson et al. (*141*)). Melting curve analysis may also be a useful tool to differentiate environmentally relevant bacteria with highly similar target sequences, potentially allowing for high-resolution discrimination (*140*); additional studies are needed to evaluate the utility of melting curve analysis for environmental applications.

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CHAPTER 3

EXPERIMENTAL EVALUATION AND MATHEMATICAL MODELING OF MICROBIALLY ENHANCED TETRACHLOROETHENE (PCE) DISSOLUTION

3.1 Abstract

Experiments to assess metabolic reductive dechlorination (chlororespiration) at high concentrations consistent with the presence of free-phase tetrachloroethene (PCE) were performed using three PCE-to-*cis*-1,2-dichloroethene (*cis*-DCE) dechlorinating pure cultures (*Sulfurospirillum multivorans*, *Desulfuromonas michiganensis* strain BB1, and *Geobacter lovleyi* strain SZ) and *Desulfitobacterium* sp. strain Viet1, a PCE-to-trichloroethene (TCE) dechlorinating isolate. Despite recent evidence suggesting bacterial PCE-to-*cis*-DCE dechlorination occurs at or near PCE saturation (0.9 – 1.2 mM), all cultures tested ceased dechlorinating at ~0.54 mM PCE. In the presence of PCE dense nonaqueous phase liquid (DNAPL), strain BB1 and strain SZ initially dechlorinated, but TCE and *cis*-DCE production ceased when aqueous PCE concentrations reached inhibitory levels. For *S. multivorans*, dechlorination proceeded at a rate sufficient to maintain PCE concentrations below inhibitory levels, resulting in continuous *cis*-DCE production and complete dissolution of the PCE DNAPL. Comparisons of the experimental data with simulations from a novel mathematical model

suggested a bioavailability number (*Bn*) threshold value of 1.25 x 10^{-2} below which dissolution enhancement will occur with the tested cultures. These results suggest that microorganisms incapable of dechlorinating at high PCE concentrations can be active in the presence of DNAPL and enhance the dissolution of PCE from free-phase PCE.

3.2 Introduction

Chlorinated solvents, such as tetrachloroethene (PCE) and trichloroethene (TCE), are widespread contaminants that threaten environmental and human health due to extensive use, accidental release, and improper disposal (*1*). In subsurface environments, chlorinated solvents are often present as dense nonaqueous phase liquids (DNAPLs) (*1*), which can result in persistent elution of contaminant plumes (*2*). Many technologies have been evaluated for containment and treatment of chlorinated ethene plumes, including bioremediation. Anaerobic bioremediation of chlorinated ethenes relies on metabolic reductive dechlorination (i.e., [de]chlororespiration), in which specialized bacteria obtain energy for growth from dechlorination reactions. Several microbial isolates transform PCE to *cis*-1,2-dichloroethene (*cis*-DCE) (e.g., *Desulfuromonas* spp. (*3,4*), *Sulfurospirillum* spp. (*5,6*), *Geobacter lovleyi* strain SZ (*7*), *Desulfitobacterium* spp. (*8,9*), and *Dehalobacter* spp. (*10,11*)). Only members of the *Dehalococcoides* group have been shown to dechlorinate beyond *cis*-DCE to vinyl chloride (VC) and ethene (summarized in reference (*13*)). None of the *Dehalococcoides* isolates grow with all chlorinated ethenes as electron acceptors (*13*), and efficient and complete dechlorination of PCE to ethene depends on the presence of multiple dechlorinating populations.

Biostimulation and bioaugmentation have been successfully implemented in the field to promote reductive dechlorination and to contain chlorinated ethene plumes (*13*). Since the rate of contaminant dissolution from the DNAPL source zone (i.e., the DNAPL-containing origin of the plume) remains unchanged, plume containment via bioremediation does not reduce overall remediation time. Physical-chemical remediation strategies (e.g., surfactant flushing, *in situ* chemical oxidation, thermal treatment) have been developed and employed to remove contaminant mass from source zones thereby accelerating remediation times (reviewed in reference (*1*)). These remediation technologies may not significantly reduce near-term environmental risk since they are generally unable to remove all of the contaminant mass (*14*). Microbial reductive dechlorination within source zones provides an alternative method for source zone remediation that may be productive in isolation or as a "polishing" step following physical-chemical remediation (*15,16*).

Metabolic reductive dechlorination within PCE-DNAPL source zones was thought to be infeasible due to the toxicity of free-phase PCE and high PCE concentrations to dechlorinating microorganisms. The ability to grow and dechlorinate at high PCE concentrations expected near or in PCE-DNAPL source zones is not a universal characteristic of PCE-dechlorinating isolates but has been reported for at least seven PCE-dechlorinating isolates (see Table 3.1). Additional studies further indicated that mixed dechlorinating consortia can dechlorinate PCE at or near saturated concentrations (e.g., see references (*17-24*)), suggesting that biologically enhanced (bioenhanced) DNAPL dissolution is possible. In bioenhanced DNAPL dissolution, biological activity in proximity of the NAPL-water interface affects interphase mass

transfer due to (i) the increase in the driving force for PCE dissolution from the NAPL to the aqueous phase as a result of reduced PCE concentrations in the aqueous phase, and (ii) the transformation of PCE to compounds with higher aqueous phase solubilities (e.g., DCEs) (*1*). Bioenhancement of NAPL dissolution has been observed experimentally in sand column and box experiments (*19,21,25*). Further experiments with batch cultures have demonstrated the ability of some dechlorinating cultures to transform a mixed (PCE/tridecane) NAPL (*17,26*) and to achieve complete PCE-DNAPL dissolution (*20*).

For the microbial reductive dechlorination process to be widely accepted as a source zone treatment strategy applicable to a wide range of sites and environmental conditions, mechanisms controlling microbial activity in the presence of DNAPL must be elucidated. The objectives of this research were to evaluate the dechlorination performance of four pure cultures at elevated PCE concentrations and to investigate the ability of the cultures to dechlorinate in the presence of PCE DNAPL. Comparison of the experimental data with simulations of a novel mathematical model developed by Dr. John A. Christ (U.S. Air Force Academy; see reference (*27*)) were also performed to provide insight into the relationship between rate-limited mass transfer from the DNAPL and aqueous phase dechlorination kinetics.

Table 3.1 Summary of reported PCE concentrations inhibitory to select reductively dechlorinating isolates and enrichment cultures.

PCE Dechlorinating Isolate	Dechlorination Activity	Reference	PCE Inhibition (mM)[a]	Aqueous Phase PCE Concentrations Tested (mM)	Method of PCE Addition
Dehalobacter restrictus strain PER-K23	PCE to *cis*-DCE	(*11*)[b] (*10*)	>0.2[c]	–[d]	PCE in HD[e]
Desulfuromonas chloroethenica strain TT4B	PCE to *cis*-DCE	(*3*)	>0.1	0-0.19 [f]	PCE in HD[g]
Desulfuromonas michiganensis strain BB1	PCE to *cis*-DCE	(*4*)	Saturation[h,i]	0.16-0.83[j], Saturation[k]	PCE in HD, Neat PCE[l]
Desulfuromonas michiganensis strain BRS1	PCE to *cis*-DCE	(*4*)	Saturation[h,i]	0.16-0.83[j], Saturation[k]	PCE in HD, Neat PCE
Sulfurospirillum multivorans	PCE to *cis*-DCE	(*6*)	>0.3	0-0.45	Neat PCE
Sulfurospirillum halorespirans	PCE to *cis*-DCE	(*5*)	–[h]	–[d]	PCE in HD
Desulfitobacterium sp. PCE1	PCE to TCE	(*8*)	–[h]	0-0.1	PCE in HD
Desulfitobacterium sp. PCE-S	PCE to *cis*-DCE	(*28*)	–[h]	–[d]	PCE in HD
Desulfitobacterium sp. TCE1	PCE to *cis*-DCE	(*29*)	–[h]	–[d]	PCE in HD
Desulfitobacterium sp. strain Y51	PCE to *cis*-DCE	(*9*)	Saturation[h]	0.0006-0.96	–[m]
Desulfitobacterium metallireducens	PCE[n], TCE[n]	(*30*)	Saturation[h]	1	–[m]
Culture "*Clostridium bifermentans* strain DPH-1"	PCE to *cis*-DCE	(*12*)	Saturation[h]	0.006-0.9	–[m]
		(*31*)[b]	0.9[o]	0.006-Saturation[p]	–[m]

Table 3.1 (continued) Summary of reported PCE concentrations inhibitory to select reductively dechlorinating isolates and enrichment cultures.

PCE Dechlorinating Isolate	Dechlorination Activity	Reference	PCE Inhibition (mM)[a]	Aqueous Phase PCE Concentrations Tested (mM)	Method of PCE Addition
Enterobacter agglomerans strain MS-1	PCE to *cis*-DCE	(*32*)	1[q]	0.01-Saturation[p]	Neat PCE
Dehalococcoides ethenogenes strain 195	PCE to ethene[r]	(*33*)	Saturation[h]	0.2-1[s]	–[m]
		(*34*)[b]	–[h]	~0.002-0.33	Varied[t]
		(*35*)[b]	–[h]	~0.33	Neat PCE
		(*36*)[b]	Saturation[h]	~0.25-1[s]	Neat PCE
PCE Dechlorinating Mixed Culture	**Dechlorination Activity**	**Reference**	**PCE Inhibition (mM)[a]**	**Aqueous Phase PCE Concentrations Tested (mM)**	**Method of PCE Addition**
PM culture	PCE to ethene	(*22*)	Saturation[h]	0.092-1.13	Neat PCE
EV Culture	PCE to ethene	(*22*)	Saturation[h]	0.039-1.06	Neat PCE
Rice/Nebraska Culture[u]	PCE to ethene	(*17*)	Saturation[h]	0.18-Saturation	–[m]
–[v]	PCE to ethene	(*18*)	–[h]	0.012-0.6	PCE in Influent[w]
KB-1/PCE	PCE to ethene	(*23*)	0.8	0.1-0.8	PCE in Methanol[x]
–[v]	PCE to ethene	(*24*)	Saturation[h]	0.05-1.2	Varied[y]
–[v]	PCE to ethene	(*20*)	Saturation[z]	0.26-Saturation[p]	Neat PCE

Table 3.1 (continued) Summary of reported PCE concentrations inhibitory to select reductively dechlorinating isolates and enrichment cultures.

[a] Concentration of PCE at or above which inhibition occurs
[b] Enrichment culture from which the isolate was obtained
[c] As reported by Damborsky (*37*) and Sharma and McCarty (*32*). The inhibitory concentration was not reported by (*10,11*).
[d] Not given
[e] PCE dissolved in hexadecane (HD)
[f] Aqueous phase concentrations calculated based on partitioning between the headspace, aqueous phase, and hexadecane phase (*3*)
[g] Some experiments in this study also added PCE from a saturated aqueous phase stock solution
[h] Dechlorination occurred at the highest concentration tested, and therefore does not reflect an inhibitory concentration
[i] The lag time before onset of PCE dechlorination increased as aqueous PCE concentrations approached saturation
[j] Aqueous phase concentration calculated based on partitioning between the headspace, aqueous phase, and hexadecane phase (*4*)
[k] Saturated PCE concentrations were assumed since DNAPL was present
[l] Undiluted (neat) PCE in free-phase (DNAPL) form
[m] Method of PCE addition not specified
[n] The endpoint of PCE and TCE dechlorination was not reported (*30*)
[o] Dechlorination of PCE to TCE occurred at 0.3 and 0.9 mM during the 7-day period monitored; *cis*-DCE was not formed
[p] Given concentrations are above the solubility limit of PCE
[q] 1 mM is listed as an aqueous phase concentration. Growth, but not dechlorination, was observed in the presence of PCE DNAPL (i.e., 2-10 mM PCE)
[r] Dechlorination of VC to ethene is cometabolic.
[s] The concentrations listed represent nominal concentrations (i.e., total mass of PCE added normalized to the aqueous phase or culture vessel volume) and may not accurately reflect dissolved phase concentrations [see references (*34,35*)]
[t] Method of PCE addition included i) addition from a saturated, aqueous PCE stock solution, ii) addition of PCE dissolved in methanol, or iii) addition of neat PCE depending on desired concentration
[u] The culture was a consortium comprised of the Rice culture (90% [vol/vol]) and the Nebraska culture (10% [vol/vol]) (*17*)

Table 3.1 (continued) Summary of reported PCE concentrations inhibitory to select reductively dechlorinating isolates and enrichment cultures.

[v] The mixed dechlorinating culture was not given a specific name

[w] The column in the experiment was fed basal salts medium containing PCE (*18*)

[x] Addition of PCE dissolved in methanol

[y] Method of PCE addition included i) addition from a saturated, aqueous PCE stock solution and ii) addition of neat PCE depending on desired concentration

[z] The presence free-phase PCE DNAPL and saturated PCE concentrations increased the lag time of PCE dechlorination and decreased the extent of dechlorination (i.e., *cis*-DCE was the main dechlorination end product as opposed to ethene)

3.3 Materials and Methods

3.3.1 Chemicals

PCE (≥99.9%) and TCE (≥99.5%) were purchased from Sigma-Aldrich Co. (St. Louis, Missouri). *cis*-DCE (99.9%) and *trans*-1,2-dichloroethene (*trans*-DCE, 99.9%) were obtained from Supelco Co. (Bellefonte, Pennsylvania). Methanol (≥99.9%) was obtained from Fisher Scientific (Hampton, New Hampshire). All of the other chemicals used were reagent grade or better unless otherwise specified.

3.3.2 Cultures and Medium Preparation

The following pure cultures were used in this study: *Desulfuromonas michiganensis* strain BB1 (DSM 15941, (*4*)), *S. multivorans* (DSM 12446, (*5,6*)), *Geobacter lovleyi* strain SZ (DSM 17278, (*7*)), and *Desulfitobacterium* strain Viet1 (*38,39*). Reduced anaerobic mineral salts medium was prepared as described (*40*) with the following modifications: KH_2PO_4, 0.2 mM; resazurin, 1 μM; $Na_2S{\cdot}9H_2O$, 0.05 mM (unless otherwise noted); dithiothreitol, 0.5 mM; anhydrous L-cysteine hydrochloride, 0.22 mM; and TES (*N*-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid), 10 mM. Vitamins (200-fold concentrated) and KH_2PO_4 (200 mM) were added from sterile, anoxic stock solutions after autoclaving. The final vitamin concentrations were as described (*40*), except vitamin B_{12} was at twice the concentration. Serum bottles (160 mL nominal capacity, Wheaton Co., Millville, New Jersey) were prepared with a N_2/CO_2 (80%/20% [vol/vol]) headspace and sealed with Teflon-lined, gray butyl rubber septa (#1014-4937, West Pharmaceuticals, Lionville, Pennsylvania) and aluminum crimp caps (Wheaton).

PCE was added as described below, sterile hydrogen gas was added via syringe using a 25 gauge needle (where indicated), and acetate or pyruvate were added to individual bottles from sterile, anoxic stock solutions using a syringe using a 25 gauge needle. The final aqueous volume following all amendments was 101 ± 1 mL.

3.3.3 Determination of Dissolved-Phase PCE Tolerance

In this study, PCE tolerance is defined as the ability to dechlorinate dissolved-phase PCE completely to the strain-specific dechlorination end product (i.e., TCE, *cis*-DCE). In the dissolved-phase PCE tolerance experiments, culture vessels received PCE dissolved in a water miscible carrier phase (i.e., methanol) to facilitate the immediate availability of dissolved-phase PCE to the microorganisms. Anoxic methanol was prepared by bubbling N_2 through methanol for 15-20 minutes in a 20 mL vial. Vials were subsequently sealed with Teflon-lined septa, secured with an aluminum crimp cap, and stored at 4ºC until use. Anoxic stock solutions of PCE dissolved in methanol were prepared inside a glove box (Coy Laboratory Products, Ann Arbor, Michigan) that contained a mixture of 95% N_2 and 5% H_2 [vol/vol]. Anoxic methanol (15 mL) was dispensed into replicate 20 mL vials. Neat PCE (350 μL) was added to one set of vials, and the vials were immediately sealed with Teflon-lined septa, secured with aluminum crimps, and stored at 4ºC until use.

Bottles containing medium were amended with the anoxic PCE/methanol stock solution (0.2 mL) and allowed to equilibrate for 2 days, resulting in measured initial aqueous phase PCE concentrations of 240-310 μM after equilibration (i.e., partitioning between the headspace and aqueous phases). Electron donors and/or carbon sources were

added as described above, were in excess throughout the experiment, and varied for each culture: acetate (5 mM) for strain BB1, acetate (5 mM) and hydrogen (10 mL) for *S. multivorans* and strain SZ, and pyruvate (5 mM) and hydrogen (10 mL) for strain Viet1. Each culture was initiated with a 5% (vol/vol) inoculum from a dechlorinating culture grown with PCE (0.33 mM) as the electron acceptor. After significant dechlorination (>80%) of the initial amount of PCE, a series of bottles received different volumes from the PCE/methanol stock solution (aqueous phase PCE concentrations after equilibration are given in parentheses): 0.2 mL (295 ± 10 μM), 0.4 mL (560 ± 45 μM), 0.6 mL (830 ± 15 μM), 0.8 mL (1,050 ± 5 μM), or 1.0 mL (1,200 ± 100 μM). The aqueous methanol concentration was adjusted to 1.2% (vol/vol)) in all bottles. Exploratory experiments demonstrated that the final methanol concentration did not affect culture performance (i.e., toxicity), pH, or analyte partitioning. Cultures were immediately shaken by hand and sampled after amendment with the PCE/methanol solution and/or pure methanol. Cultures were incubated upright at 22°C on a platform shaker at 175 rpm.

3.3.4 Dechlorination Studies in the Presence of PCE DNAPL

Dechlorination studies in the presence of PCE DNAPL were performed with strain BB1, strain SZ, and *S. multivorans.* Electron donors and/or carbon sources were added as described above and varied for each culture: acetate (5 mM) for strains BB1 and SZ and pyruvate (5 mM) and hydrogen (5 mL) for *S. multivorans*. In these experiments, the concentration of $NaHCO_3$ was increased to 60 mM to increase the buffering capacity, and the concentrations of Na_2S and L-cysteine were increased to 0.1 mM and 0.27 mM, respectively. Serum bottles containing medium were amended with

0.2 mL of the anoxic PCE/methanol stock solution and allowed to equilibrate for 2 days to yield initial aqueous phase PCE concentrations of approximately 300 μM. The bottles received a 5% (vol/vol) inoculum of a dechlorinating culture grown with PCE (0.33 mM) as the electron acceptor. Triplicate cultures of strain BB1, strain SZ, and *S. multivorans* were incubated at 22°C and shaken upright at 150 rpm on a platform shaker. Following complete dechlorination of the initial PCE amendment, the cultures were amended with an additional 0.2 mL of the anoxic PCE/methanol stock solution. After complete dechlorination of dissolved-phase PCE, a 500 μL gastight syringe (#1750, Hamilton Co., Reno, Nevada) was used to amend the bottles with 50 μL of neat PCE to form a single PCE droplet (DNAPL). Control cultures were inactivated by the addition of sterile O_2 gas (10 mL) after growth of *S. multivorans* on dissolved-phase PCE but before amendment of PCE DNAPL. Additions of hydrogen gas (10 mL at Day 2, 5 mL for all other amendments) to the cultures of *S. multivorans* were performed on Days 2, 4, 5, 6.5, 7, 10, 12, and 15 after DNAPL addition. The experiment with *S. multivorans* was repeated in duplicate vessels shaken at increased agitation (175 rpm), which was assumed to increase the rate of PCE dissolution from the DNAPL phase. The *cis*-DCE production following DNAPL addition was determined by subtracting the *cis*-DCE aqueous-phase concentration present before DNAPL addition from the *cis*-DCE concentrations measured following DNAPL addition.

3.3.5 Analytical Methods

Chlorinated ethenes were quantified via gas chromatography. Aqueous-phase samples (1 mL) were removed via sterile syringe and 25 gauge needle and injected into a

20-mL headspace vial previously sealed with a Teflon-lined gray butyl septum secured with an aluminum crimp cap. To maintain positive pressure, sterile nitrogen or hydrogen (1 mL) was injected into each culture vessel to replace the volume removed during sampling. Sample analysis was performed on a Hewlett-Packard (HP) 7694 headspace autosampler and a HP 6890 gas chromatograph (GC) equipped with a HP-624 column (60 m by 0.32 mm; film thickness, 1.8 μm nominal) and a flame ionization detector (FID). The following conditions for the autosampler were determined to be optimal for chlorinated ethene concentration measurements: oven temperature, 70ºC; vial equilibration time using maximum agitation, 15 min; sample loop (1 mL) temperature, 125ºC; transfer line (80 cm) temperature, 125ºC; pressurization time, 0.5 min; loop fill time, 0.03 min; loop equilibration time, 0.05 min; inject time, 0.5 min; carrier gas pressure, 2 psig; vial pressurization, 10 psig. Based on the GC conditions described below, the cycle time on the autosampler was set to 14 min. Helium was used as the carrier gas for both the autosampler and the GC. The GC inlet was maintained at 200°C and 23.11 psig with a total flow of 5.7 mL/min, and the inlet split ratio was 0.1:1. The oven temperature for the GC was kept at 60°C for 2 minutes followed by an increase at a rate of 25°C/min until the oven reached 200°C. The column was operated in the constant flow mode at 3.0 mL/min at 23.11 psig. The FID detector was operated at a temperature of 280°C with the following gases (flow rates in parentheses): hydrogen (30 mL/min), air (400 mL/min), and helium (27 mL/min). The method detection limits for chlorinated ethenes were <2.5 μM. Standards for chlorinated ethenes were prepared as described (*41,42*). Briefly, a known amount of analyte dissolved in methanol was added to bottles containing medium. Standards for PCE contained only PCE, while standards for TCE,

cis-DCE, and *trans*-DCE contained all three chlorinated ethenes. Distribution of each chlorinated ethene between the headspace and aqueous phases was determined using Henry's constants specific for each chlorinated ethene (*41*).

3.3.6 Model Development

A novel mathematical model applicable to batch cultures, which accounts for loss of dechlorinating activity at inhibitory PCE concentrations and simultaneously describes PCE-DNAPL dissolution and reductive dechlorination kinetics, was developed by Dr. John A. Christ (U.S. Air Force Academy) to simulate the experimental data described herein. Please see reference (*27*) for a complete description of the model. Simulation results are reproduced here with permission of Dr. Christ for comparison of the experimental data with the simulated results. These comparisons were performed in an effort to provide insight into the relationship between rate-limited mass transfer from the DNAPL and aqueous phase dechlorination kinetics.

3.4 Results

3.4.1 Dissolved-Phase PCE Tolerance

Experiments conducted with dissolved-phase PCE indicated that all four pure cultures tested were unable to dechlorinate when aqueous PCE concentrations exceeded ca. 540 μM. Figure 3.1 shows representative results for the dechlorination performance of *Desulfuromonas michiganensis* strain BB1 in the presence of dissolved-phase PCE at tolerable and intolerable concentrations. All cultures were established with low PCE

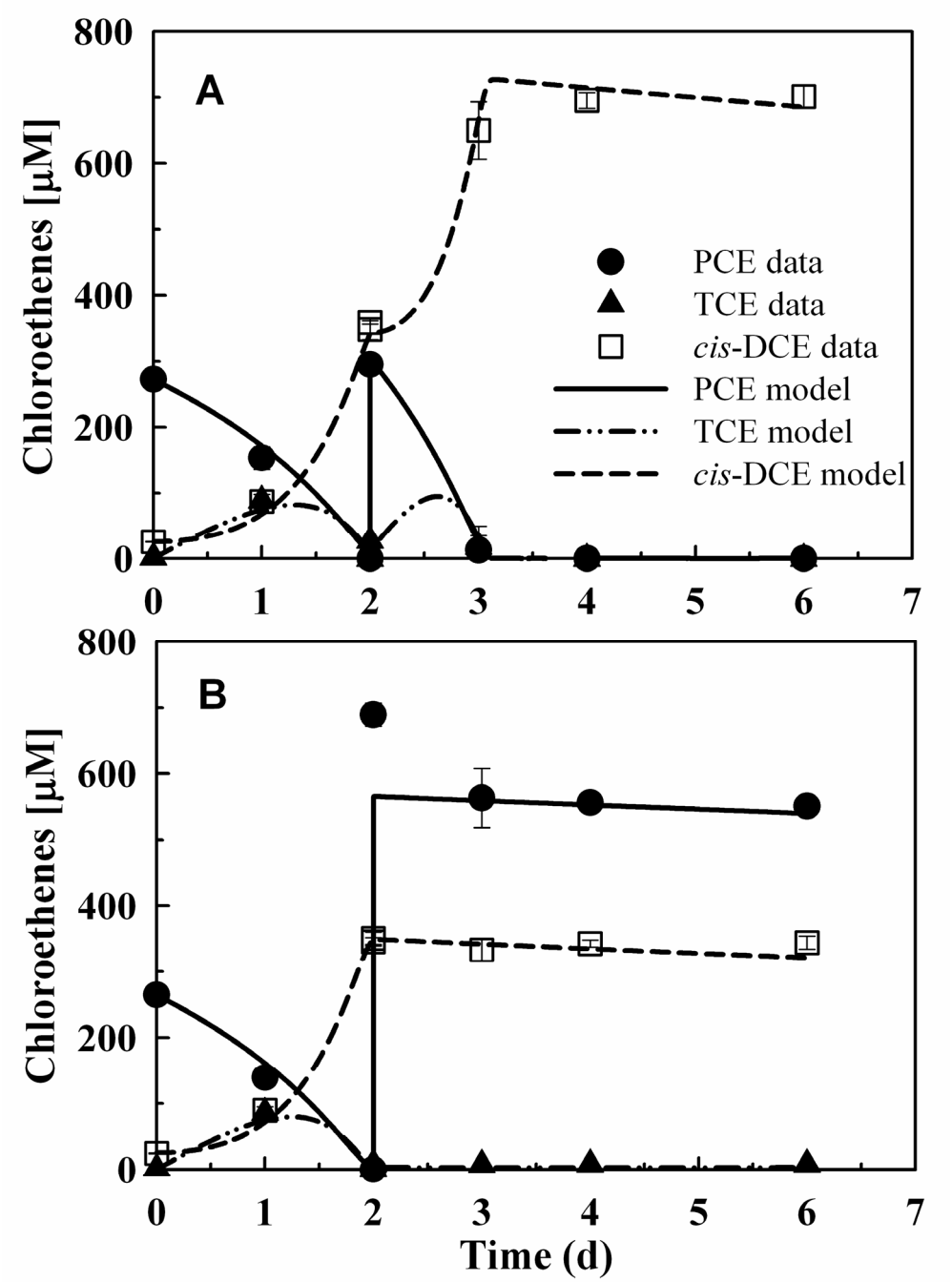

Figure 3.1 Dechlorination performance of *Desulfuromonas michiganensis* strain BB1 at various PCE concentrations (see Materials and Methods section for details). Low PCE concentrations were used for all initial conditions (Days 0-2), followed by amendment of additional PCE to tolerable (A) or intolerable (B) PCE concentrations. The legend is shared between A and B. All data points represent average values from triplicate cultures, and error bars represent one standard deviation. If no error bars are shown, the standard deviations were too small to be illustrated. The lines represent simulated chlorinated ethene concentrations fitted (Figure 3.1A) or predicted (Figure 3.1B) by the developed mathematical model (see reference (*27*)).

concentrations initially (Days 0-2, Figure 3.1), which was followed by PCE conversion to *cis*-DCE and trace levels (<2.1 μM) of *trans*-DCE, with the formation of TCE as an intermediate (Figures 3.1A and B). After amendment of additional PCE to a concentration of 295 ± 10 μM on Day 2, strain BB1 dechlorinated PCE to *cis*-DCE (Figure 3.1A). In a parallel experiment (Figure 3.1B), the concentration of PCE after the Day 2 addition was increased to 690 ± 10 μM. The aqueous phase concentration of PCE decreased to 560 ± 45 μM by Day 3 at which point the concentration of PCE remained relatively constant for the duration of the experiment. The initial decrease in PCE between Days 2 and 3, which also occurred when higher PCE concentrations were tested (data not shown), was attributed to partitioning of PCE from the aqueous phase to the headspace. Formation of TCE and accumulation of *cis*-DCE did not occur (Figure 3.1B, Days 2-6), indicating that strain BB1 did not dechlorinate PCE at the tested concentration. Additional experiments at higher PCE concentrations confirmed that strain BB1 could not dechlorinate PCE at concentrations >560 μM (data not shown).

Results obtained for the other three pure cultures tested were similar in that dechlorination ceased or was severely inhibited at PCE concentrations ≥540 μM (data not shown). *S. multivorans* and *Geobacter lovleyi* strain SZ dechlorinated PCE to *cis*-DCE at PCE concentrations ≤330 μM but did not dechlorinate PCE to *cis*-DCE at PCE concentrations ≥540 μM. Limited dechlorination (<25% of the initial PCE mass) of PCE to TCE occurred with strain SZ at PCE concentrations of ca. 540 μM (13 day incubation, TCE production ceased on Day 8; data not shown). *Desulfitobacterium* sp. strain Viet1 dechlorinated PCE to TCE at PCE concentrations ≤330 μM, while only limited (<5%)

accumulation of TCE occurred at PCE concentrations of ca. 540 μM; PCE dechlorination did not occur with strain Viet1 at PCE concentrations >540 μM.

3.4.2 Dechlorination in the Presence of PCE DNAPL

The dechlorination performance of *Desulfuromonas michiganensis* strain BB1 in the presence of PCE DNAPL is shown in Figure 3.2. Strain BB1 dechlorinated dissolved-phase PCE (two amendments of 300 μM each) completely to *cis*-DCE and trace levels of *trans*-DCE (<4 μM) (data not shown), before the addition of PCE DNAPL. After the addition of PCE DNAPL, PCE dissolution resulted in accumulation of PCE in the aqueous phase (Figure 3.2). Initially (before Day 2 following DNAPL addition), the cultures accumulated TCE, *cis*-DCE, and trace amounts of *trans*-DCE (~155, ~1,050, and <5 μM, respectively). Dechlorination ceased after 1.4 days when the PCE concentration reached 470 ± 130 μM (Figure 3.2). PCE continued to accumulate in the aqueous phase until equilibrium conditions (1,070 ± 40 μM) were reached around Day 6. DNAPL was visible at the conclusion of the experiment. Similar results were observed with strain SZ in the presence of PCE DNAPL, although greater amounts of TCE (310 ± 25 μM) and *cis*-DCE (1,260 ± 180 μM) accumulated in the aqueous phase after addition of PCE DNAPL (data not shown).

The dechlorination performance of *S. multivorans* in the presence of PCE DNAPL is shown in Figure 3.3. *S. multivorans* dechlorinated dissolved-phase PCE (two amendments of 300 μM each) completely to *cis*-DCE and trace levels of *trans*-DCE (<5 μM) (data not shown), before the addition of PCE DNAPL. For the inactivated controls, PCE dissolution from the DNAPL phase resulted in equilibrium conditions and

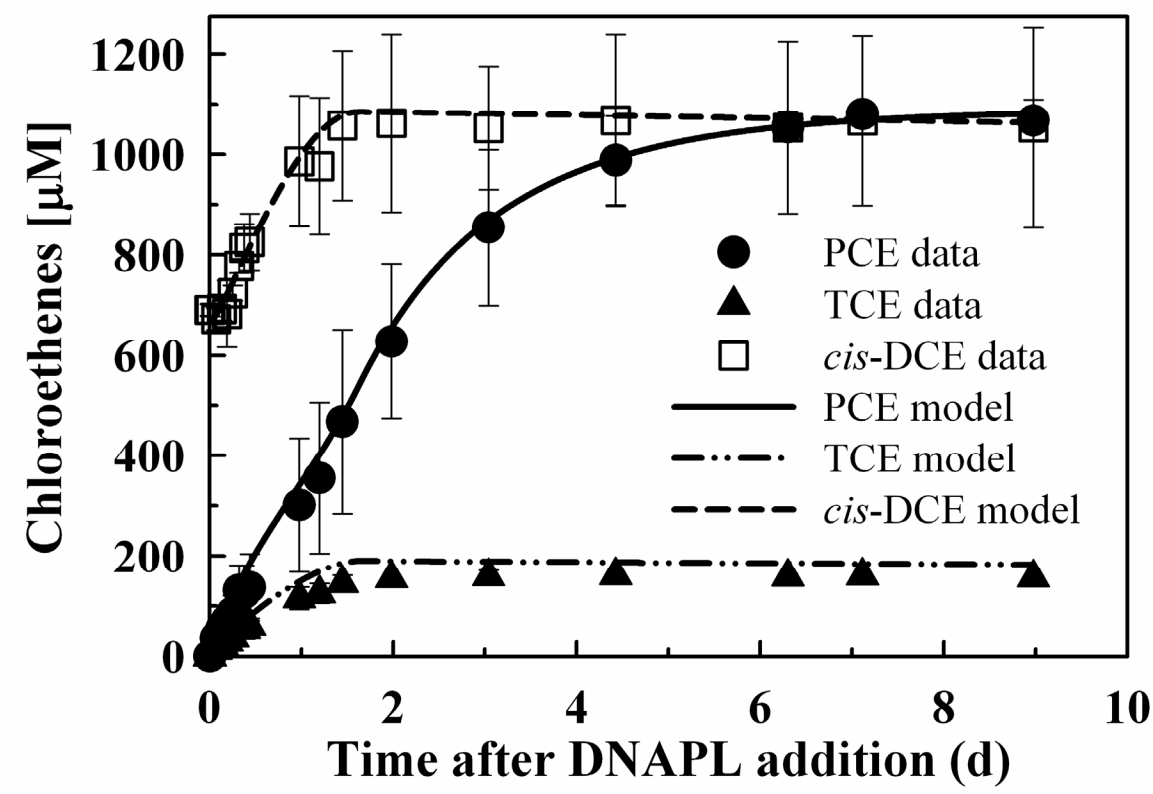

Figure 3.2 Dechlorination performance of *Desulfuromonas michiganensis* strain BB1 in the presence of PCE DNAPL. All data points represent average values from triplicate cultures, and error bars represent one standard deviation. If no error bars are shown, the standard deviations were too small to be illustrated. The lines represent simulated chlorinated ethene concentrations predicted by the developed mathematical model (see reference (*27*)).

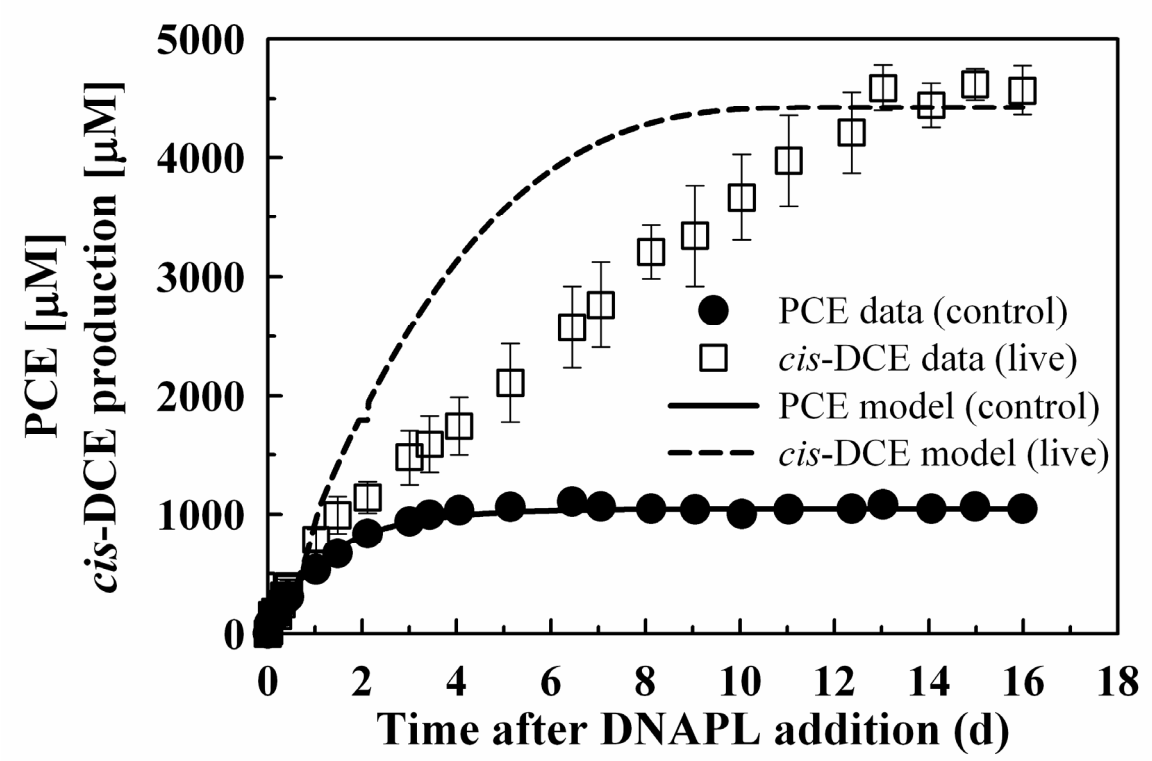

Figure 3.3 Dechlorination performance of *Sulfurospirillum multivorans* in the presence of PCE DNAPL. *cis*-DCE production was calculated by subtracting the *cis*-DCE aqueous-phase concentration present before DNAPL addition from the *cis*-DCE aqueous-phase concentration measured following DNAPL addition and is presented to aid in comparing the initial rate of *cis*-DCE accumulation to the rate of PCE dissolution in the inactivated controls. All data points represent average values from triplicate cultures shaken at 150 rpm, and error bars represent one standard deviation. If no error bars are shown, the standard deviations were too small to be illustrated. The lines represent simulated chlorinated ethene concentrations fitted (inactivated control data) or predicted (live data) by the developed mathematical model (see reference (*27*)).

saturated PCE concentrations (1,040 ± 40 μM) around Day 4 (Figure 3.3). In the inactivated controls, dechlorination did not occur and the DNAPL droplet remained visible throughout the entire experiment. In live cultures, *S. multivorans* dechlorinated PCE and produced *cis*-DCE (Figure 3.3) and *trans*-DCE (data not shown). *cis*-DCE and *trans*-DCE accumulated until Day 13, at which time the production of *cis*-DCE and *trans*-DCE leveled off at 4,600 ± 200 μM and 28 ± 2 μM, respectively. Unlike similar experiments with strain BB1 (Figure 3.2) and strain SZ (data not shown), PCE did not accumulate in the aqueous phase when *S. multivorans* was grown in the presence of PCE DNAPL. PCE was detected in six of the twelve samples taken before or on Day 3, five of which were at levels <6 μM, while TCE was only detected in two samples at concentrations <40 μM. PCE and TCE were not detected in any sample after Day 3. The DNAPL droplet decreased in size (visual observation) throughout the experiment and was completely dissolved after 13 days in the live cultures, corresponding to the time at which *cis*-DCE production ceased. When the experiment with *S. multivorans* was repeated with an increased agitation speed (175 rpm), which presumably increased the rate of PCE dissolution from the DNAPL, transient accumulation of PCE and TCE to maximum aqueous phase concentrations of 124 and 118 μM was observed at 3 and 6 h after DNAPL addition, respectively (Figure 3.4).

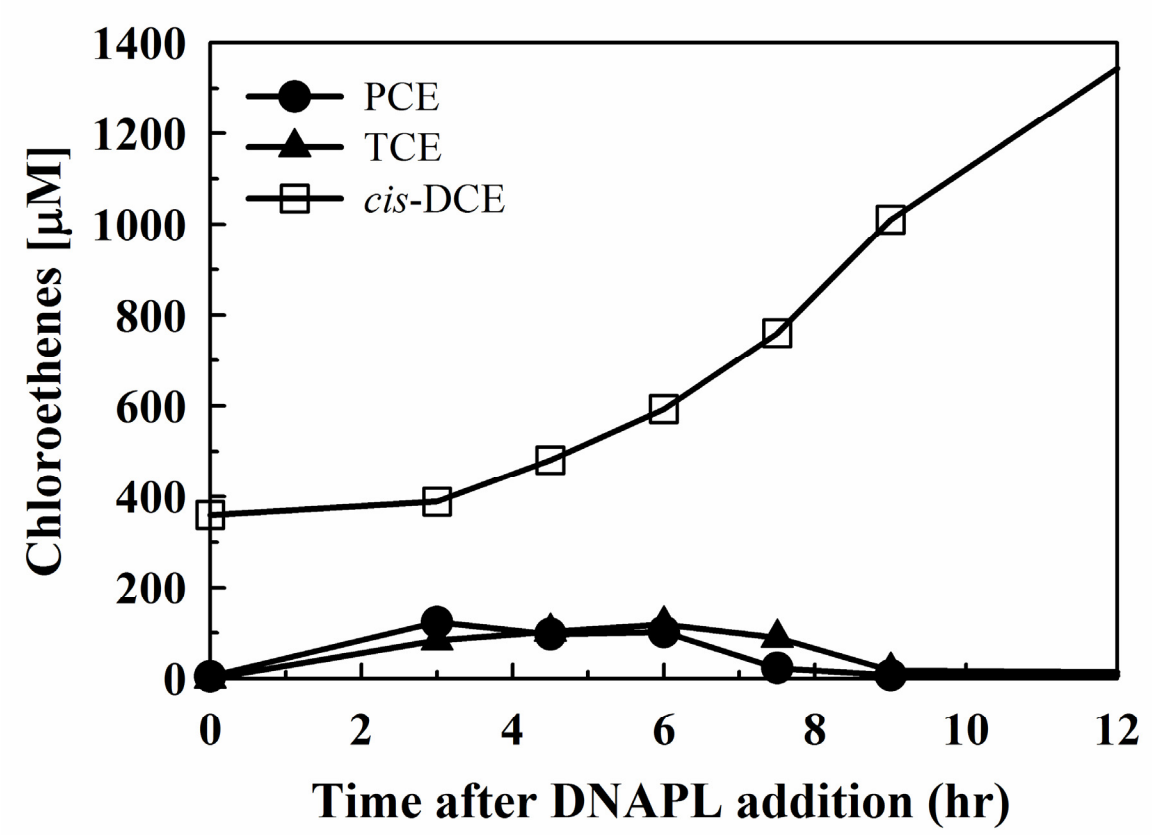

Figure 3.4 Dechlorination performance of *Sulfurospirillum multivorans* in the presence of PCE DNAPL at an increased agitation speed. The results for a single, representative culture shaken at 175 rpm show the transient accumulation of PCE and TCE at early times (<12 hr).

3.4.3 Comparison of Experimental Results with Model Simulations

Model simulations of dissolved phase chlorinated ethene concentrations are shown in Figures 3.1 – 3.3. Kinetic coefficients for reductive dechlorination were obtained by fitting the model to measured dissolved-phase chlorinated ethene concentrations for strain BB1 (Figure 3.1A) and *S. multivorans* (data not shown) using the optimization method as described (*27*). The results shown in Figure 3.1 demonstrate that the fitted model provided an accurate representation of the concentration data from the dissolved-phase experiments. Incorporation of substrate inhibition (modeled after Luong (*43*)) to account for loss of dechlorinating activity at inhibitory PCE concentrations correctly predicted the cessation of dechlorinating activity when PCE concentrations approached the experimentally derived inhibitory PCE concentration (i.e., ~540 μM for strain BB1, Figure 3.1B). Unlike other substrate inhibition models (e.g., Haldane) that have been used successfully to model the gradual decline in transformation rates as substrate concentration increases (*22*), experimental results presented herein suggest that dechlorination ceased when PCE concentrations exceeded a maximum tolerable level for the tested cultures. The selected substrate inhibition model better represents this terminal inhibition concentration and eliminates the need to quantify another kinetic parameter (i.e., inhibition constant) (*43*). Differences between the simulated and experimental PCE concentrations following amendment of additional PCE on Day 2 (Figure 3.1B) stem from the model assumption that equilibrium between the gas and aqueous phases occurred instantaneously, while the experimental results indicate a short period (<1 day) of nonequilibrium. The gradual decline of the simulated *cis*-DCE and PCE concentrations after Day 3 (Figure 3.1A and B, respectively) is due to the model

assumption that losses due to sampling occurred throughout the entire experiment rather than at discreet sampling times.

The model accurately predicted dechlorination in the presence of PCE DNAPL using (i) the best-fit kinetic coefficients obtained from the dissolved-phase simulations, (ii) a best-fit mass transfer coefficient for PCE through the aqueous-phase boundary layer (κ^{PCE} = 1.84 ± 0.03 m/d) obtained by fitting the model to PCE dissolution from the DNAPL in the inactivated control (Figure 3.3), and (iii) the experimentally derived PCE concentration in the aqueous phase at equilibrium with the organic phase ($C_{ao}^{PCE\text{-}e}$ = 1043 ± 2 μM). For strain BB1, the model predicted PCE dechlorination to TCE and *cis*-DCE before accumulation of PCE in the aqueous phase to inhibitory concentrations (~540 μM), as was observed experimentally (Figure 3.2). Furthermore, the model correctly predicted that the dechlorination activity of *S. multivorans* would prevent significant PCE accumulation, resulting in accumulation of *cis*-DCE and ultimately the complete dissolution of the PCE DNAPL, as was observed experimentally (Figure 3.3). Although the simulated rate of *cis*-DCE accumulation slightly exceeded the observed value, the simulated and experimental time to complete dissolution of the PCE DNAPL was approximately the same (13 days after DNAPL addition). The difference between the measured and predicted rates of *cis*-DCE accumulation could be attributed to (i) assumptions used to model dissolution (e.g., diminishing sphere model), (ii) changes in the dissolution rate due to microbial growth on or near the surface of the DNAPL (*44*), (iii) inhibition of microbial activity at increasing *cis*-DCE concentrations (*4,17*), and/or (iv) changes in PCE dissolution rates from the NAPL due to partitioning of *cis*-DCE into the NAPL (*17,26*).

3.5 Discussion

Microbially enhanced PCE-DNAPL dissolution has been suggested as a productive PCE and TCE source zone remedy (*17-21,25,26*). Several studies with pure cultures (see Table 3.2) and with mixed dechlorinating consortia (e.g., (*17-24*)) have shown that bacterial PCE dechlorination occurs at or near saturated PCE concentrations. For instance, *Desulfuromonas michiganensis* strain BB1 was reported to dechlorinate at saturated PCE concentrations and to grow in the presence of PCE DNAPL (*4*). The results presented here demonstrate that strain BB1 cannot dechlorinate at PCE concentrations above 540 μM, and similar observations were made for other PCE-to-TCE and PCE-to-*cis*-DCE dechlorinating organisms (i.e., *Sulfurospirillum multivorans*, *Geobacter lovleyi* strain SZ, and *Desulfitobacterium* sp. strain Viet1). The discrepancies in the inhibitory PCE concentrations determined here for strain BB1 and previous results (*4*) are likely due to differences in the experimental methodology including (i) the use of Teflon-lined septa as opposed to butyl rubber stoppers to minimize contaminant loss due to sorption, and (ii) the addition of PCE in a soluble carrier phase (i.e., methanol) to ensure rapid PCE dissolution and availability to the organisms. In the study by Sung et al. (*4*), PCE was added undiluted (i.e., neat) or in a separate-phase insoluble carrier (i.e., hexadecane). The addition of PCE using these techniques is appropriate as long as adequate time is given to ensure equilibrium conditions before initiation of the experiment; otherwise, initial conditions will be at nonequilibrium, yielding PCE concentrations that could be significantly below targeted values. DNAPL dissolution is a slow process, and time is required to establish equilibrium conditions (see Figure 3.3).

The inhibitory PCE concentrations reported in this study for microbial isolates of different phylogenetic lineages accurately reflect dissolved-phase PCE concentrations and demonstrate that the cultures tested cannot dechlorinate PCE at or near the aqueous solubility of PCE. Similar, carefully conducted studies should be performed with other PCE-dechlorinating bacteria (see Table 3.1) to elucidate culture-specific inhibitory PCE concentrations.

Although the bacterial isolates included in this study failed to dechlorinate at saturated PCE concentrations, these organisms impacted the dissolution and transformation of PCE from free-phase DNAPL. Microbial activity in the presence of PCE DNAPL occurred as long as aqueous PCE concentrations remained below inhibitory concentrations (i.e., the system was not in equilibrium). Such activity might explain one mechanism by which biologically enhanced dissolution occurs with dechlorinating microorganisms incapable of dechlorinating at high or saturated PCE concentrations. The ability to dechlorinate in the presence of DNAPL, resulting in complete DNAPL dissolution and transformation, is likely not restricted to *S. multivorans* but may be observed with the other tested dechlorinating organisms (i.e., strains BB1 and SZ) under the appropriate conditions (e.g., higher biomass concentrations, decreased PCE dissolution rates, attenuation of PCE to non-inhibitory levels due to other processes [e.g., sorption, dispersion]).

In the experiments, the detection of low, transient PCE concentrations in the presence of PCE DNAPL indicated that the dechlorination rate in cultures of *S. multivorans* was equal to, or possibly limited by, the PCE dissolution rate from the

DNAPL. The bioavailability number (*Bn*) has been proposed for quantifying the relationship between the rate of dissolution and the rate of biotransformation (*45*):

$$Bn = \frac{\kappa^{PCE} a'_{ao}}{k_{\max}^{PCE} X_0 / K_S^{PCE}}$$

where a'_{ao} (L^{-1}) is the initial specific interfacial area of the DNAPL (interfacial area normalized by aqueous phase volume), $k_{\max}{}^{PCE}$ (M/M_x-T) is the maximum utilization rate for PCE, $K_S{}^{PCE}$ (M/L^3) is the half-saturation constant for PCE, X_o (M_x/L^3) is the cell titer (i.e., biomass concentration) of the PCE-to-*cis*-DCE dechlorinating bacteria at the beginning of DNAPL dissolution, and all other values are as described above. *Bn* is a dimensionless parameter that arises during the derivation of the Best equation, which assumes the mass transfer flux and substrate degradation are approximately equal (quasi-steady-state) (*45*). A *Bn* less than unity represents dissolution-controlled bioavailability, while a *Bn* greater than unity represents conditions where transformation is limited by the catalyst. Using the best-fit dechlorination and dissolution rates determined using the numerical model, *Bn* for the batch experiments was determined to be 1.56×10^{-2} and 6.97×10^{-3} for strain BB1 and *S. multivorans*, respectively. The results presented here suggest that dechlorinating isolates and mixed cultures incapable of dechlorinating at high or saturated PCE concentrations can continuously dechlorinate in the presence of a DNAPL, leading to dissolution enhancement relative to abiotic conditions, when conditions corresponding to a $Bn < 1.25 \times 10^{-2}$ (e.g., *S. multivorans*) exist. For such dechlorinating cultures, conditions corresponding to a *Bn* greater than this threshold value may not lead to sustained dissolution enhancement and PCE transformation because the catalyst (e.g., strain BB1) will be inhibited as PCE accumulates. Application of the

reported *Bn* threshold to cultures that tolerate saturated PCE concentrations or have different culture-specific inhibitory PCE concentrations should be performed cautiously. A sensitivity analysis found that the *Bn* threshold ranged from 1.0 to 2.0 x 10^{-2} when the culture-specific inhibitory PCE concentration was varied from 25 to 75% of the PCE aqueous-phase solubility, respectively.

DNAPL dissolution in aquifer formations is a complex process that depends on many variables, including the DNAPL saturation distribution, DNAPL interfacial area, porous media heterogeneity, and groundwater flow. The mass transfer coefficient obtained in the experimental system falls within the range of field mass transfer coefficients predicted by commonly employed correlations (e.g., 0.13 – 4 m/d (*46*)) and is consistent with grid-scale mass transfer coefficients used in multidimensional numerical simulators (e.g., UTCHEM, MISER). Although many of the correlations predict equilibrium aqueous phase contaminant concentrations at the local scale under most environmental conditions (e.g., (*47*)), dispersive transport and dilution are relevant processes in source zones with heterogeneous NAPL distributions. Dispersive transport and dilution effects may reduce concentrations below levels that are inhibitory to some dechlorinating organisms. Dechlorination activity in zones with tolerable PCE concentrations would further reduce aqueous phase contaminant concentrations and sustain enhanced dissolution of downgradient NAPL ganglia or pools because of the increased mass transfer driving force. Therefore, microbial activity may enhance dissolution in PCE-DNAPL source zones even if the dechlorinating bacteria cannot tolerate high PCE concentrations or are not active in the immediate vicinity of the NAPL-water interface. Future experiments are needed to address the influence of source zone

DNAPL architecture and microbial distribution within DNAPL source zones on dissolution processes.

The inability of the tested bacteria to dechlorinate PCE at concentrations >540 μM does not imply that this is a universal characteristic of PCE dechlorinators, and the present results should not be used to discount bioremediation as a viable remedial option at sites where PCE concentrations exceed 540 μM. Several mixed culture studies suggest that PCE dechlorination occurs at high, even saturated, concentrations of PCE (e.g., (*17-24*)). The ability of these mixed cultures to dechlorinate at elevated PCE concentrations may be due to (i) the presence of dechlorinating strains acclimated to high PCE concentrations, or (ii) dechlorinators that exhibit increased PCE tolerance as members of mixed microbial communities (e.g., biofilms). For example, an increased tolerance to high levels of PCE may be related to the presence of large cell aggregates (i.e., granules), which characterize some dechlorinating consortia (see Chapter 6, Section 6.4.2). The aqueous-phase concentration that dechlorinating organisms experience within such granules may be below inhibitory levels and lower than bulk (i.e., measured) concentrations due to diffusional limitations or sorption of PCE to cells on the outside of the aggregates. A similar protection mechanism from high concentrations of PCE may also occur within aquifer material biofilms, similar to what has been observed for other stressors (*48*). In many bacteria, environmental stressors (e.g., heat) regulate the production of a large number of proteins that aid in bacterial survival, and such general stress response systems are important for microbial tolerance to other solvents (e.g., toluene) (reviewed in reference (*49*)). Recently, interspecies communication following stress exposure and the regulation of stress responses have been shown to result in stress-

adapted phenotypes (*50,51*). At present, the complex interactions among dechlorinators and other microbial populations are poorly understood. Elucidating these interactions, including their potential roles in avoiding PCE inhibition and enhancing dechlorination activity in the presence of PCE DNAPL, is likely to further promote source zone bioremediation.

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CHAPTER 4

QUANTITATIVE REAL-TIME PCR (qPCR) CORRELATES MICROBIAL ACTIVITY AND DISTRIBUTION WITH ENHANCED CONTAMINANT DISSOLUTION FROM A PCE-NAPL SOURCE ZONE: PART 1 – EXPERIMENTS WITH *SULFUROSPIRILLUM MULTIVORANS*

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4.1 Introduction

Chlorinated solvents, such as tetrachloroethene (PCE), are common groundwater contaminants that are frequently encountered as dense nonaqueous phase liquids (DNAPLs) in aquifer formations (*1*). Subsurface environments that contain DNAPL represent long-term threats to environmental and public health since contaminant elution from these source zones can occur for decades (*2*). Microbial reductive dechlorination has recently emerged as a promising approach for the remediation of chlorinated ethene DNAPL source zones, either in isolation or as a polishing step for the removal of residual DNAPL remaining after physical-chemical treatment (*1-4*). In metabolic microbial reductive dechlorination, specialized bacteria obtain energy for growth from reductive dechlorination reactions that detoxify the parent compound to benign ethene (i.e., [de]chlororespiration). Several microbial isolates transform PCE to *cis*-1,2-dichloroethene (*cis*-DCE) (e.g., *Sulfurospirillum multivorans* (*5,6*); for a complete list of

isolates, see references (*7,8*)). Only members of the *Dehalococcoides* group have been shown to dechlorinate beyond *cis*-DCE to vinyl chloride (VC) and ethene (summarized in (*9*)). Efficient and complete dechlorination of PCE to ethene, therefore, depends on the presence of multiple dechlorinating organisms (*9*).

Microbial reductive dechlorination within PCE-DNAPL source zones was once thought to be infeasible due to the toxicity of free-phase PCE and high concentrations of PCE to dechlorinating microorganisms (as reviewed in Chapters 2 and 3). Recently, dechlorinating isolates and microbial consortia were reported to dechlorinate PCE at or near saturated concentrations (see Chapter 3, Table 3.1), suggesting that source zone bioremediation is feasible. Several experiments in continuous-flow stirred-tank reactors, one-dimensional (1-D) columns, two-dimensional (2-D) aquifer cells, and three-dimensional (3-D) simulated aquifers demonstrated that the microbial reductive dechlorination process can result in biologically enhanced (bioenhanced) DNAPL dissolution (1.3 – 14 fold increased dissolution compared to abiotic processes alone) (*10-17*). Such enhancement may remove significant amounts of source zone contaminant mass, reduce source zone longevity, and decrease near- and long-term environmental and health risks.

Although current evidence suggests that bioremediation may be an effective source zone remediation technology, the formation of toxic intermediates and only minimal production of benign ethene were observed in the previous studies (*10-17*). Several reasons for incomplete PCE dechlorination have been proposed: insufficient contact/residence times (*16*), low system pH (*12,18*), inhibitory levels of polychlorinated ethenes (i.e., PCE, TCE, and *cis*-DCE) (*16,19,20*), and inadequate supply of electron

donor (*15,16,21*). Although complete detoxification of PCE to ethene is desirable, partial dechlorination is still beneficial for reducing source zone longevity and increasing the bioavailability of dechlorination products (*cis*-DCE, VC) to down-gradient processes (e.g., anaerobic biobarriers (*22,23*), aerobic metabolism (*24,25*)).

Although the results of the previous bioenhanced dissolution studies suggested dechlorination activity in the vicinity of PCE-DNAPL and microbial colonization of the source zone by dechlorinating organisms, few of the previous studies evaluated microbial community dynamics and composition during bioenhanced dissolution. Adamson et al. (*10*) used denaturing gradient gel electrophoresis (DGGE) and polymerase chain reaction (PCR) to evaluate changes in microbial diversity and the presence of *Dehalococcoides* spp., respectively, on a limited number of samples from their simulated aquifer. DGGE indicated reduced microbial diversity in the simulated aquifer as compared to the bioaugmentation inoculum, while *Dehalococcoides* spp. were detected in the two samples collected from the simulated aquifer. Sleep et al. (*13*) also used DGGE as well as quantitative real-time PCR (qPCR) to gather microbiological information during bioenhanced dissolution. After bioaugmentation of the 2-D aquifer cell, the number of *Dehalococcoides* cells detected in the effluent increased by approximately 3 orders-of-magnitude, corresponding to a significant decrease in PCE concentrations and an equivalent increase in PCE dechlorination products (mainly *cis*-DCE). Interestingly, the highest number of *Dehalococcoides* organisms (10^7 cells per gram soil) was detected in samples taken nearest the DNAPL source zone. Although microbial growth was observed throughout the aquifer cell, spatial heterogeneity of *Dehalococcoides* spp. was observed. Given the limited number of sampling locations (5 sampling ports in the 76 cm

x 38 cm x 2.54 cm aquifer cell), the spatial heterogeneities could not be characterized with sufficient resolution, especially the distribution of dechlorinators in relationship to the PCE-DNAPL source zone. To further explore the relationship between microbial distribution and dissolution enhancement, experiments described herein were performed in continuous-flow, 1-D columns to evaluate the dechlorination performance and microbial distribution of *S. multivorans*, a PCE-to-*cis*-DCE dechlorinator, in the immediate vicinity of PCE-NAPL. *S. multivorans* was selected for evaluation in the 1-D column experiments due to its ease of handling (robustness) and the fact that it was observed to dechlorinate in the presence of PCE-DNAPL (see Chapter 3).

4.2 Materials and Methods

4.2.1 Chemicals

HPLC-grade tetrachloroethene (PCE) was purchased from Fisher Scientific (Fair Lawn, New Jersey). Hexadecane (HD; 99% purity) was obtained from Sigma-Aldrich Co. (St. Louis, Missouri). HD (226.43 g/mol) is insoluble in water and has a liquid density of 0.77 g/mL (*26*). Oil-Red-O ($C_{26}H_{24}N_4O$), a hydrophobic dye, was obtained from Fisher Scientific. For analytical standard curves, PCE (≥99.9%) and TCE (≥99.5%) were purchased from Sigma-Aldrich Co., and *cis*-DCE (99.9%) and *trans*-1,2-dichloroethene (*trans*–DCE, 99.9%) were obtained from Supelco Co. (Bellefonte, Pennsylvania). All of the other chemicals used were reagent grade or better.

4.2.2 NAPL Preparation

Two different PCE-NAPLs were used in the column experiments: pure PCE-DNAPL and a mixed NAPL. The mixed NAPL, comprised of PCE and HD, was utilized (i) to simulate a PCE-containing NAPL consisting of a recalcitrant organic fraction (HD) with low aqueous solubility and (ii) to reduce equilibrium dissolved-phase PCE concentrations to below those inhibitory for *S. multivorans* (< 540 μM [90 mg/L]; see Chapter 3). The PCE/HD mixed NAPL was prepared in an anoxic glove box (Coy Laboratory Products, Ann Arbor, Michigan) filled with 95% N_2 and 5% H_2 [vol/vol] by diluting anoxic, N_2-flushed PCE into anoxic, N_2-flushed HD to a final ratio of 0.25/0.75 (mol/mol) (e.g., 10.4 mL PCE and 89.6 mL HD). Since the equilibrium aqueous phase solubility of pure PCE is ~200 mg/L (~1,200 μM) (*26*), the equilibrium aqueous phase solubility of PCE residing within the mixed NAPL was theoretically 50 mg/L (~300 μM). Exploratory experiments conducted in batch vessels verified the solubility of the mixed NAPL. The density of the mixed NAPL was determined to be 0.86 g/mL using a 2-mL pycnometer (Ace Glass Incorporated, Vineland, New Jersey) as described (*27*); the density of pure PCE DNAPL is 1.625 g/mL (*28*).

4.2.3 Medium Preparation and Column Inocula

Reduced, anaerobic, mineral salts medium with a low chloride (LC medium) content was prepared as described (*29*), except for changes to the concentrations of the following components: $Na_2S \cdot 9H_2O$, 1 mM; anhydrous L-cysteine hydrochloride, 0.2 mM; $NaHCO_3$, 60 mM; and resazurin, 2 μM. TES (*N*-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid) was also included at a final concentration of 10 mM.

Vitamins (200-fold concentrated) and sodium/potassium phosphate (Na_2HPO_4/KH_2PO_4; 200 mM) were added from sterile, anoxic stock solutions after autoclaving. The final vitamin concentrations were as described (*30*), except vitamin B_{12} was at twice the concentration. Pyruvate was added to a final concentration of 20 mM (where indicated) from an anoxic, filter-sterilized stock solution using a syringe and a 25 gauge needle. Pyruvate was added in excess so that electron donor limitations did not control dechlorination activity or bioenhanced dissolution. Reduced medium (1 L) was prepared in 2-liter Pyrex medium bottles (Fisher Scientific), each of which was capped with a sidearm plug (19 mm i.d.; Wheaton Co., Millville, New Jersey) and a modified 25-mL anaerobic culture (Balch) tube (Bellco Glass Inc., Vineland, New Jersey) sealed with a black butyl-rubber stopper (Geo-Microbial Technologies, Inc., Ochelata, Oklahoma) and an aluminum crimp cap (Wheaton). The flasks contained a N_2/CO_2 (80%/20% [vol/vol]) headspace.

Sulfurospirillum multivorans (DSM 12446, (*5,6*)), a PCE-to-*cis*-DCE dechlorinating, strictly anaerobic pure culture, was used in this study. Cultures of *S. multivorans* (1 L) were grown with 20 mM pyruvate as the sole carbon and energy source to serve as inocula for the column experiments (*6*). Before the column was packed (see Section 4.2.5.1), samples of the inocula were collected for DNA extraction and quantitative real-time PCR (qPCR) analysis.

4.2.4 Column Design and Construction

A 1-D borosilicate glass column (Standard Chromaflex Column) that included Teflon end plates was purchased from Kontes Glass Company (Vineland, New Jersey).

The overall dimensions of the column were 60 cm in length by 4.8 cm inside diameter (i.d.). As shown in the schematic of the 1-D column system (Figure 4.1), the column was retrofitted with eleven sampling side ports, located on alternating sides at 5 cm intervals along the entire length of the column. The port nearest to the column influent (5 cm down-gradient) was designated Port 1, and the ports were numbered sequentially with increasing distances from the influent. Port 11, therefore, was 55 cm down-gradient of the influent and 5 cm up-gradient of the column exit. The side ports allowed for determination of the spatial distribution of *S. multivorans* and chlorinated ethenes. The side ports were constructed by the glass shop at the Georgia Institute of Technology; each side port consisted of the upper portion of a glass screw-cap HPLC vial (Fisher Scientific) permanently attached to the column via a glass capillary tube (8 mm outside diameter [o.d.]; 2 mm i.d.). The column was spatially separated into three regions: a ~10 cm long source zone containing PCE-NAPL, a ~10 cm long transition zone directly down-gradient of the source zone, and a 40 cm long plume region down-gradient of the source zone. Ports 1 and 2 were within the source zone, and Ports 5-11 were in the plume region. Due to some fingering of the imbibed NAPL (see Section 4.2.5.3), Ports 3 and 4 were in a transition zone between the source zone and the plume region.

An influent system was designed to operate continuously at flow rates of < 1 mL/min and to maintain anaerobic conditions. The influent medium reservoir (2-liter medium bottle, see Section 4.2.3) was connected to the column via 0.32 cm o.d. stainless steel tubing and a three-way valve (Hamilton Co., Reno, Nevada) at the bottom of the column. Based on flow and volume of medium remaining, the reservoir was routinely replaced with another reservoir containing freshly prepared medium. Since medium was

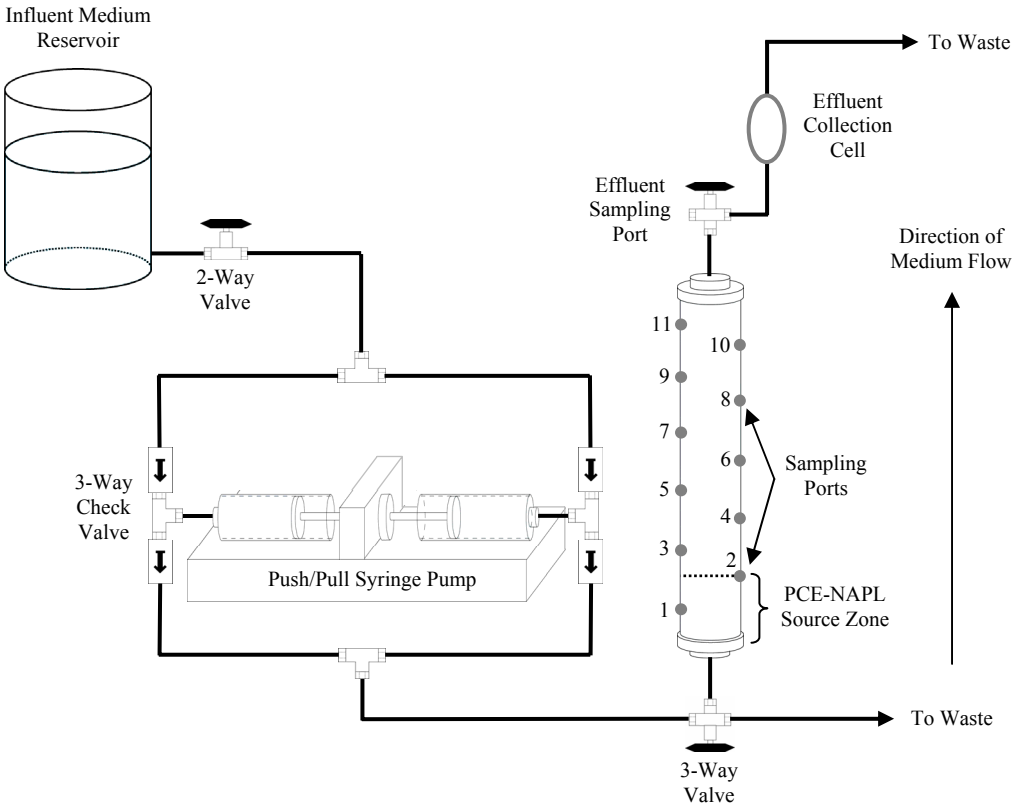

Figure 4.1 Schematic diagram of the 1-D column system (modified and adapted from Suchomel (*27*)).

continuously withdrawn from the reservoir, the reservoir was pressurized daily to 15 psig with sterile N_2 to prevent intrusion of air into the anoxic influent system. Medium was pumped from the influent reservoir to the column via two 25 mL gas-tight syringes (Hamilton) using a syringe pump (model PHD 2000 push/pull, Harvard Apparatus, Holliston, Massachusetts). The selected pump was capable of simultaneous infusion and withdrawal, allowing for continuous operation (i.e., one syringe was infusing medium into the column, while the other was withdrawing medium from the influent reservoir). The column was operated in an up-flow manner, (i.e., flow proceeded from the bottom of the column to the top). As flow exited the column, it entered an in-line, custom-built effluent collection cell. The effluent collection cell had an approximate volume of 25 mL and was constructed from glass tubing connected to the column via a four-way 90° valve (Hamilton) and Teflon tubing (0.32 cm o.d.).

4.2.5 Column Operation

Two column experiments were performed: one with the mixed-NAPL and one with the pure PCE-DNAPL. Both column experiments operated independently (i.e., at separate times). This section describes the operation of the 1-D column experiments, which occurred in multiple stages. For both column experiments, the column was packed with porous medium and a culture of *S. multivorans* as the resident aqueous phase (see Column Setup, Section 4.2.5.1). After the column was packed, reduced medium was continuously flushed through the column to monitor the elution (i.e., washout) of the added microorganisms (see Microbial Elution Phase, Section 4.2.5.2). Following the microbial elution phase, the column was imbibed with the mixed or pure NAPL (see

NAPL Imbibition, Section 4.2.5.3). After a residual NAPL source zone was established in the column, effluent and side port samples were periodically collected to monitor bioenhanced dissolution (see NAPL Dissolution Phase, Section 4.2.5.4). At the conclusion of each experiment, operation of the column ceased, and the column was destructively sampled (see Column Dissection, Section 4.2.5.5).

4.2.5.1 Column Setup. For both column experiments, the column was packed with autoclaved, N_2-flushed Federal Fine Ottawa sand as the porous medium. Federal Fine (30-140 mesh) Ottawa sand (U.S. Silica Company, Berkeley Spring, West Virginia) was used as representative porous medium for the 1-D column experiments due to its very low organic carbon content (< 0.1 g organic content per kg solid) (*27*) and light color, which allowed for easy observation of dyed NAPL within the column. Characteristics (e.g., mean grain size, intrinsic permeability, uniformity index, particle size distribution) of Federal Fine Ottawa sand have been described elsewhere (*27*).

For each experiment, the column was packed under liquid-saturated conditions in approximately 2 cm sections with a culture of *S. multivorans* as the resident aqueous phase (see Section 4.2.3 for a description of the inocula for the column experiments). Packing occurred inside a system of glove bags continuously flushed with nitrogen to maintain anoxic conditions. Exploratory experiments determined that the glove bag system was sufficient to maintain anoxic conditions, limiting exposure of the *S. multivorans* culture to air (oxygen). Use of the microbial culture as the residence aqueous phase, as opposed to culture injection into the column (i.e., column bioaugmentation), ensured a nominally-uniform initial distribution of *S. multivorans*

within the column. After packing, the column remained in the N_2-flushed glove bag system for approximately 24 hours; during this period, the column was maintained with no flow. The pore volume (PV) of each column was estimated to be 397 mL and 400 mL for the mixed-NAPL and pure PCE-DNAPL experiments, respectively. The PV was estimated by subtracting the sand volume within the column from the total system volume (1090.9 mL). The mass of dry sand added to the column was determined by mass difference between dry sand before column setup and the remaining sand after column setup (i.e., the sand that was not packed within the column). The sand volume added to the column was then calculated by dividing the sand mass added to the column by the particle density of the sand (2.65 g/mL). Given this PV, the hydraulic retention (residence) time of the column was approximately 1.1 days at the target (operational) flow rate of 0.25 mL/min.

4.2.5.2 Microbial Elution Phase. The column was connected to the influent system after removal from the glove bag system. Reduced medium (without pyruvate) was then continuously flushed through the column at 0.25 mL/min (nominal) for 3.3 and 4.3 PV for the pure PCE-DNAPL and mixed-NAPL column experiments, respectively, to monitor the elution (i.e., washout) of the added *S. multivorans* cells. The medium did not contain a carbon source or electron donor (i.e., pyruvate), nor did the column yet contain PCE-NAPL; therefore, minimal growth and/or decay of *S. multivorans* were assumed in the microbial elution phase. Effluent samples (10-20 mL) were taken periodically from the effluent collection cell for DNA extraction and qPCR analysis.

4.2.5.3 NAPL Imbibition. After the microbial elution phase, the column was imbibed with the anoxic, mixed or pure PCE-NAPL (see Section 4.2.2) through the three-way valve located at the bottom of the column. During imbibition, the valve was connected to a 100-mL gas-tight glass syringe (Hamilton) using 0.32 cm (o.d.) Teflon tubing. A syringe pump (model 22, Harvard Apparatus) was used to inject NAPL into the column at a constant flow rate of 1 mL/min until the NAPL source zone saturated the first 10 cm (nominal) of the column. NAPL injection was then terminated, and the column was operated in a down-flow manner (1 mL/min) for approximately one hour to remove mobile NAPL and establish a source zone with residual (entrapped) NAPL ganglia. For operation of the column in the down-flow manner, the influent system was temporarily connected to the four-way valve at the top of the column. At the conclusion of down-flow operation, the source zones for the mixed NAPL and pure PCE-DNAPL experiments contained approximately 14 mL and 9 mL of NAPL, respectively, corresponding to an organic phase saturation of approximately 0.21 and 0.11 over the first 10 cm of the column. Although the NAPL distribution within the source zone was relatively uniform in both column experiments, some fingering of the NAPL was apparent. The fingering was not factored into the determination of residual saturation, which may have resulted in an erroneously high estimate of source zone NAPL saturation. The pure PCE-DNAPL source zone had less NAPL fingering and was more uniform than the mixed NAPL source zone.

4.2.5.4 NAPL Dissolution Phase. For both column experiments, the column was continuously operated and dechlorination activity monitored for a period of up to several

weeks after NAPL imbibition. During this time, the influent contained 20 mM pyruvate, and the influent system operated at 0.25 mL/min (hydraulic retention [residence] time of approximately 1.1 days). Effluent samples (~20 mL) were collected every 1-2 days from the effluent collection cell using 20 mL syringes connected to the four-way valve between the column and the collection cell (see Figure 4.1). The effluent samples were divided into separate sub-samples for chlorinated ethenes, pH, and microbial analyses. For the mixed NAPL column experiment, the side ports were sampled for chlorinated ethenes after 5, 11, and 17.5 PV of flushing, and additional 1-mL side port samples were taken from each port for microbial analysis following the 17.5 PV sampling event. In order to minimize disruption of aqueous flow within the column during side port sampling, samples were taken at a constant rate (0.1 mL/min) with a 2.5 mL gas-tight syringe (Hamilton) connected to a syringe pump (model 22, Harvard Apparatus) operating in withdrawal mode.

4.2.5.5 Column Dissection. At the conclusion of each experiment, the column was returned to the N_2-flushed glove bag system and destructively sampled (sectioned) into six 10-cm long cores. The cores were recovered from the column by sequentially driving aluminum cylinders (3.8 cm o.d.) into the uppermost exposed 10 cm of column material. The top of the cylinders were sealed, and the cores, which remained encased in the aluminum cylinders, were removed from the column and frozen overnight (-20°C) to prevent additional microbial activity and redistribution of the liquid phases. Each core was then thawed, removed intact from the aluminum cylinder under sterile conditions,

and further divided into sections 2 cm in length. These subsections were homogenized and stored at -20°C until genomic DNA extraction (see Section 4.2.7).

4.2.6 Abiotic Experiments

A series of independent abiotic column experiments, described elsewhere (*27*), were performed to characterize the physical-chemical properties of the column (e.g., hydrodynamic dispersion). Two non-reactive tracer (0.01 M potassium iodide) experiments were performed in the 1-D column to assess the hydrodynamic dispersion within the packed column without *S. multivorans* or NAPL (*27*). Both tracer experiments indicated uniform flow through the column and the absence of physical nonequilibrium. Simulated breakthrough curves (BTC software, version 4.2) were comparable to the experimental breakthrough curves (*27*). Based on the results of the simulations, the Peclet number (an indicator of system ideality) of the column was estimated to range between 590 ± 150 and 850 ± 120 and the dispersivity was estimated to range between 0.07 ± 0.01 cm and 0.10 ± 0.02 cm. The overall pore volume of the packed column predicted by the simulations was estimated to range between 390 and 400 mL, which was consistent with experimental estimates of the pore volume (397 – 400 mL).

In a third abiotic experiment, the mixed NAPL was imbibed into the column to evaluate abiotic PCE dissolution from the mixed NAPL (*27*). At the target flow rate for the biotic columns (0.25 mL/min), the dissolved phase concentrations in the abiotic experiment were similar to those expected from equilibrium considerations (300 μM, 50 mg/L; see Section 4.2.2) along the entire length of the column (see reference (*27*)), indicating the absence of abiotic mass transfer limitations.

4.2.7 DNA Extraction

Biomass was collected periodically from 10-20 mL effluent by centrifugation at 4°C for 30 minutes at 3,220 × g. All but ~1 mL of the supernatant was decanted, and the bacterial cell pellet was suspended in the remaining supernatant. Centrifugation was then repeated at 16,000 × g at room temperature for 10 minutes. For the side port samples, ~1 mL liquid samples were centrifuged at 16,000 × g at room temperature for 10 minutes. The supernatant from all bacterial cell pellets was removed, and the pellets were stored at -20°C until genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, California) following the modifications described by Ritalahti et al. (*31*). DNA was obtained in a final volume of 200 µL buffer AE (provided with the QIAamp DNA Mini Kit) and stored at -20°C until qPCR analysis. DNA was also extracted from select solid-phase samples (~1 g column material [i.e., wet sand]) using the UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Inc., Solana Beach, California). The selected solid-phase samples were chosen to correspond to the same locations as the aqueous phase side ports. DNA was obtained in a final volume of 50 µL of solution S5 (provided with the UltraClean Soil DNA Isolation Kit) and stored at -20°C until qPCR analysis.

4.2.8 Quantitative Real-Time PCR (qPCR) Analysis

qPCR analysis to quantify the *S. multivorans* was performed using SYBR Green-based detection chemistry and primers (SpSm1f, 5'-TCGTTGCAGGTATCGCTATG-3'; SpSm1r, 5'-TTCAACAGCAAAGGCAACTG-3') that target the *pceA* reductive dehalogenase gene of *S. multivorans* (*32*). The specificity of the primer pair was verified by BLAST analysis and experimentally confirmed with the outlined qPCR protocol with

DNA from the select reductively dechlorinating species (*Dehalococcoides* sp. strain BAV1, *Dehalobacter restrictus*, *Desulfuromonas michiganensis* strain BB1, *Desulfitobacterium* sp. strain Viet1, *Geobacter lovleyi* strain SZ, and *Anaeromyxobacter dehalogenans* strain 2CP-C). The following qPCR protocol was developed and optimized as part of this study and is the first qPCR protocol designed for quantitative detection of *S. multivorans*. The qPCR reaction mixture contained 15 µL of QuantiTect SYBR Green PCR master mix (Qiagen, Valencia, California), 300 nM of each primer, and 3 µL of template DNA in a total reaction volume of 30 µL. The PCR temperature program was as follows: 2 min at 50ºC, 15 min at 95ºC, followed by 40 cycles of 15 sec at 94°C, 30 sec at 52°C, and 30 sec at 72°C. qPCR was carried out in a Applied Biosystems 7500 Fast Real-Time PCR System. Standard curves were generated following the procedure outlined in Ritalahti et al. (*31*) and used a 10-fold dilution series of quantified genomic DNA of *S. multivorans*. The initial DNA concentration was determined spectrophotometrically at 260 nm. The number of gene copies in each standard was estimated from the DNA concentrations as described by Ritalahti et al. (*31*), assuming a 1.64 Mb genome size for *S. multivorans* and one copy of the *pceA* gene per genome (G. Diekert, personal communication). The genome of *S. multivorans* has not been sequenced; therefore, the genome size was assumed to be the size of the closest sequenced member of the *Campylobacteraceae* family (*Campylobacter jenuni* (*33*)). The terms "*pceA* gene copies" and "cell numbers" are per mL of fluid and are used interchangeably. The linear range for quantification was 10^2 - 10^8 *pceA* gene copies per µL of template DNA (R^2 = 0.993; amplification efficiency = 1.89 (*34*)), resulting in a quantification limit of ~100 *pceA* gene copies per µL of template DNA. To identify false

positives, template DNA was replaced with sterile water (i.e., no template controls, NTCs).

4.2.9 Analytical Methods

Chlorinated ethenes were quantified via gas chromatography. Aqueous-phase samples (1 mL) were collected periodically and injected into 20-mL headspace vials previously sealed with Teflon-lined gray butyl septa secured with aluminum crimp caps. The samples were analyzed with a Hewlett-Packard (HP) 7694 headspace autosampler connected to a HP 6890 gas chromatograph (GC) equipped with a HP-624 column (60 m by 0.32 mm; film thickness, 1.8 μm nominal) and a flame ionization detector (FID) as described (see Chapter 3, reference (*35*)). Standard calibration curves for chlorinated ethenes analysis were prepared as described (*35-37*). The pH of column effluent was periodically determined by placing a 1-mL sample into a N_2-flushed microcentrifuge tube and using a VWR Model 8000 pH meter (#511710, VWR Scientific, West Chester, Pennsylvania) equipped with a Accumet gel-filled pH combination electrode (#13-620-290, Fisher Scientific).

4.3 Results and Discussion

4.3.1 Microbial Elution Phase

The inoculum for the mixed NAPL column experiment contained 8.0 x 10^7 *pceA* genes per mL. Taking into account this estimate and the column pore volume (397 mL), a total of 3.2 x 10^{10} *S. multivorans* cells were added to the column during packing. The

number of *pceA* genes per mL eluted from the column and recovered in the effluent during the microbial elution phase is shown in Figure 4.2A. The number of cells detected in the effluent ranged from 5.0×10^6 to 1.0×10^7 per mL and was significantly lower than the number of cells in the inoculum. Although there are a limited number of data points, the peak observed in Figure 4.2A may represent a "breakthrough" of *S. multivorans* eluting from the column.

The cumulative and percent recovery of *pceA* genes in the effluent during the microbial elution phase is shown in Figure 4.2B. Cumulative gene recovery was determined by taking the area under the curve in Figure 4.2A, while percent recovery compared the cumulative gene recovery to the total number of *pceA* genes added to the column (see above). Calculation of percent recovery assumed no growth or decay, which is reasonable since the influent medium did not contain any growth substrate during this experimental phase. As shown in Figure 4.2B, only 26% of the biomass initially present in the column was recovered in the effluent after 4.3 PV of flushing, indicating significant attachment and/or retention of *S. multivorans* within the column. Similar results were observed in the pure PCE-DNAPL column experiment. Approximately 30% of the cells added to the column in the pure PCE-DNAPL experiment were recovered in the effluent after 3.3 PV of flushing (data not shown), again suggesting that a significant fraction of the added *S. multivorans* cells was retained. The high percent of *S. multivorans* retained within the columns was unexpected. Microbial attachment and transport depends on porous media characteristics and can vary widely even among closely related bacteria (*38*). Future studies should systematically evaluate attachment and transport of *S. multivorans* and other dechlorinating organisms in porous medium to

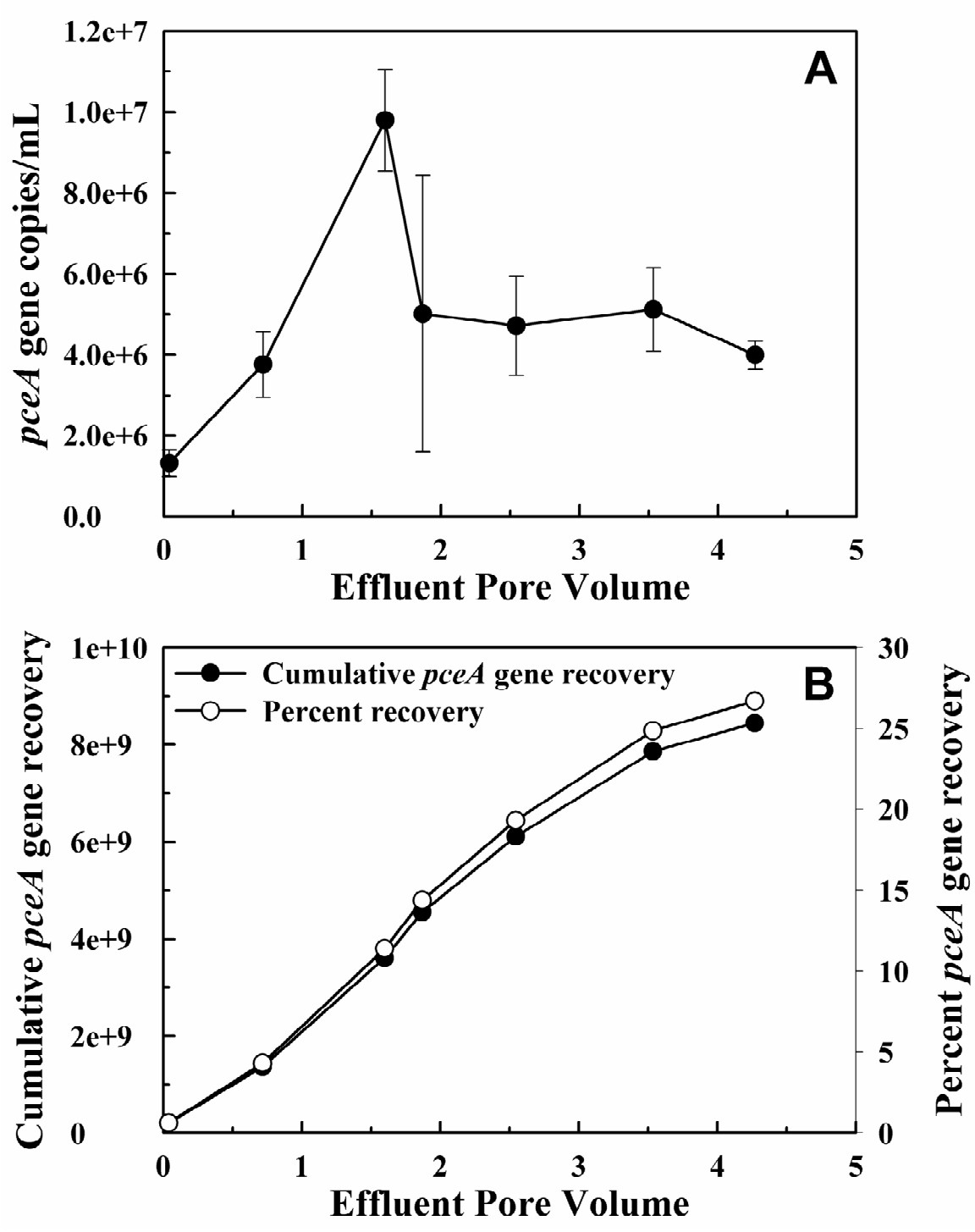

Figure 4.2 Effluent *pceA* gene copies per mL (A) and cumulative and percent *pceA* gene recoveries (B) during the microbial elution phase of the mixed-NAPL experiment.

gain a mechanistic understanding of the processes that govern microbial transport in subsurface systems.

4.3.2 NAPL Dissolution Phase of the Mixed-NAPL Column Experiment

4.3.2.1 Effluent Samples. As described above, the number of *pceA* genes per mL of effluent for the mixed-NAPL column experiment ranged from 5.0 x 10^6 to 1.0 x 10^7 before the addition of the mixed NAPL. Approximately 2 PV after NAPL addition (see Section 4.2.5.3) and the start of flushing with pyruvate-amended medium, the number of *pceA* genes detected in the effluent sharply increased from 10^6–10^7 per mL to approximately 2 x 10^9 per mL by 4 PV of flushing. This 2-3 orders-of-magnitude increase was maintained for the duration of the experiment (data not shown). Approximately 10^{13} *pceA* gene copies were recovered in the effluent over the course of the mixed-NAPL experiment. This value is substantially greater than the total number cells (i.e., *pceA* genes) added to the column initially (3.2 x 10^{10}), indicating that significant growth of *S. multivorans* occurred during the column experiment.

Effluent chlorinated ethene concentrations and pH for the mixed-NAPL column are shown in Figure 4.3. The dashed line in Figure 4.3 indicates the equilibrium dissolved-phase solubility of PCE in the mixed NAPL (~300 μM), which was determined experimentally (Section 4.2.2). In the absence of bioenhanced NAPL dissolution and assuming equilibrium mass transfer (Section 4.2.6), the total chlorinated ethene concentration (i.e., the sum of the concentrations of PCE, TCE, *cis*-DCE, and *trans*-DCE) should be equal the equilibrium value. Minimal dechlorination of PCE occurred during the initial 2 PV of flushing, which corresponds to the time when little growth of

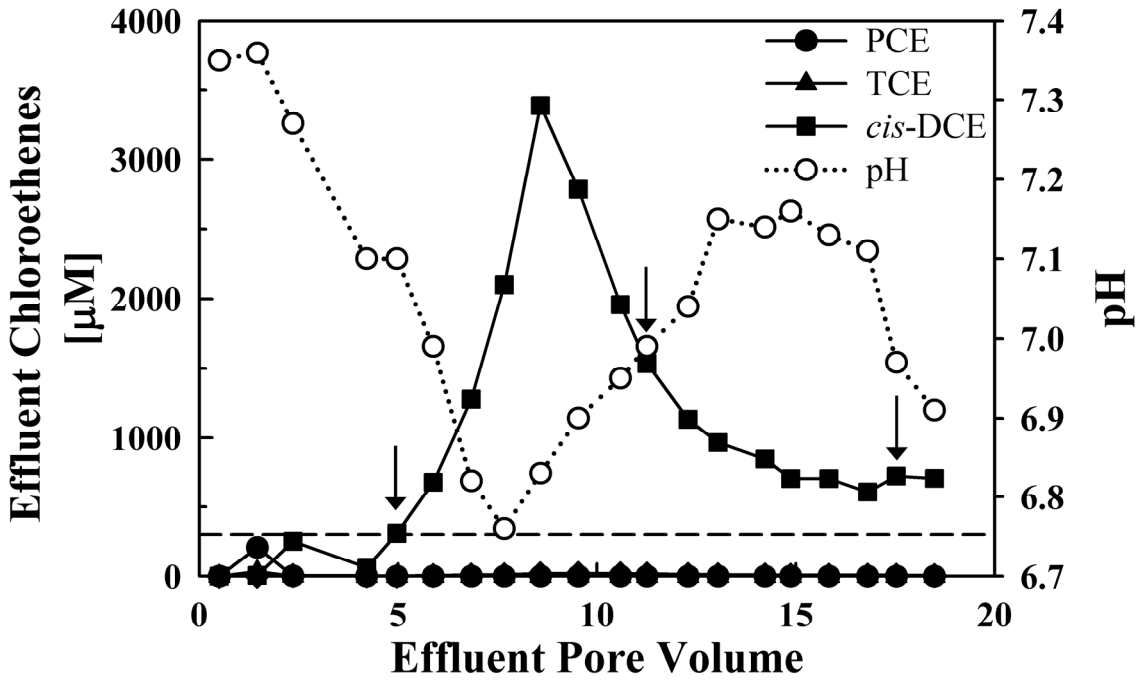

Figure 4.3 Effluent chlorinated ethene concentrations and pH measured during the NAPL dissolution phase of the mixed-NAPL column experiment. The expected chlorinated ethene concentration (~300 μM), based on equilibrium solubility of PCE in the mixed-NAPL, is shown as the dashed line (see text for details). Arrows indicate side port sampling events.

S. multivorans was observed (see above). Following this initial period and growth of *S. multivorans*, dissolved-phase PCE was completely dechlorinated to *cis*-DCE and trace amounts of *trans*-DCE. After 3 PV of flushing, PCE was no longer detected in the effluent. Initially (< 5 PV), the total chlorinated ethene concentration approached the expected value. After 5 PV of flushing, the *cis*-DCE concentrations sharply increased to a maximum value of 3,400 μM at 8.6 PV. For most of the experiment with the mixed-NAPL, *cis*-DCE concentrations were significantly higher than the expected total chlorinated ethene concentration (dashed line, Figure 4.3), indicating bioenhanced dissolution of PCE from the mixed NAPL. After 8.6 PV of flushing, the *cis*-DCE concentration declined, reaching approximately 700 μM at the conclusion of the experiment. While this pseudo-steady state value is significantly lower than the maximum *cis*-DCE concentration observed, it is nonetheless greater than twice the expected value and indicates that bioenhanced dissolution of PCE from the mixed NAPL was ongoing even at the termination of the column experiment. The decline in *cis*-DCE production and overall column performance could be due to a number of factors, including depletion of PCE from the mixed NAPL and toxicity of high concentrations of *cis*-DCE to *S. multivorans*. Another possible explanation for the reduced performance is the decrease in pH. The microbial reductive dechlorination process is an acidifying process that releases hydrochloric acid (HCl) during each sequential transformation step (see Figure 2.4). Therefore, for every mole of PCE dechlorinated to *cis*-DCE, 2 moles of HCl are produced. The relationship between reductive dechlorination, formation of HCl, and pH reduction was apparent in the mixed-NAPL column experiment (see Figure 4.3). Changes in effluent pH generally correlated with (mirrored) changes in *cis*-DCE

production (i.e., increasing *cis*-DCE concentrations resulted in decreasing pH and vice versa). Although the influent medium flushed through the column was buffered with 60 mM bicarbonate, the pH of the effluent decreased from ~7.35 to a minimum of ~6.75 when *cis*-DCE concentrations approached 3,500 µM. Reductive dechlorination by *S. multivorans* occurs optimally in a narrow pH range (7.0 – 7.5) (*6*). The pH drop in the column below 7.0, therefore, may have resulted in decreased dechlorination activity of *S. multivorans*. Adamson et al. (*18*) observed that extensive PCE dechlorination drastically lowered pH in NAPL-containing batch systems and speculated that lower pH values decreased both the rate and extent of dechlorination. Unless systems are well buffered, decreases in pH may occur in DNAPL source zones due to microbial dechlorination activity. Enhancement of NAPL dissolution and subsequent transformation to lesser chlorinated ethenes through biological activity (i.e., microbial reductive dechlorination) could lead to decreased performance of such biological systems as a result of low pH levels. Decreased bioremediation performance could also occur if other microorganisms relevant for the microbial reductive dechlorination process (e.g., *Dehalococcoides* spp., fermentors, acetogens) exhibit narrow optimal pH ranges. Future research should evaluate the effects of pH on bioenhanced dissolution.

4.3.2.2 Side Port Samples. The results from the side port samples taken during the NAPL dissolution phase of the mixed-NAPL column experiment are shown in Figure 4.4, where Port 1 is nearest the column influent and Port 11 is nearest the column exit. Ports 1 and 2 were within the source zone, and Ports 5-11 were in the plume region down-gradient of the source zone. Due to some fingering of the imbibed NAPL (Section

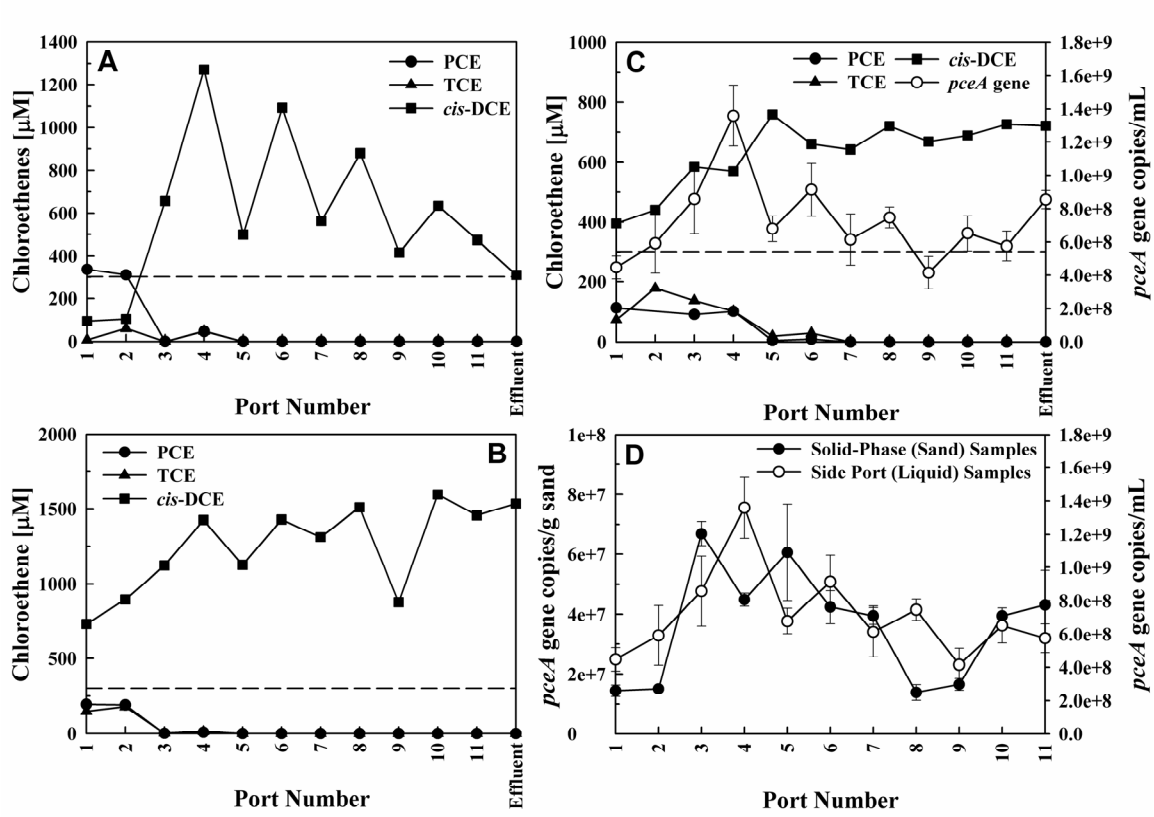

Figure 4.4 Profiles of chlorinated ethene concentrations and microbial cell numbers along the length of the 1-D column in the mixed-NAPL experiment. Side port profiles after 5, 11, and 17.5 pore volumes of flushing are shown in panels (A), (B), and (C), respectively. Effluent concentrations taken at the same time are shown for comparison. The expected chlorinated ethene concentration (300 μM) based on equilibrium solubility of PCE in the mixed-NAPL is shown as the dashed lines. Panel D compares the *pceA* gene distribution determined with side port (liquid) samples taken after 17.5 PV or solid-phase (sand) samples taken after column dissection (18.6 PV). Port 1 is nearest the influent and in the source zone while Port 11 is nearest the effluent.

4.2.5.3), Ports 3 and 4 were in a transition zone between the source zone and the plume region. The data presented in Figure 4.4 indicate dechlorination activity in the source zone since TCE, *cis*-DCE, and traces of *trans*-DCE were detected in addition to PCE. The first side port sampling event occurred after 5 PV of flushing and coincided with the onset of bioenhanced dissolution. As shown in Figure 4.4A, PCE concentrations were near the expected (equilibrium) value in the source zone (Ports 1 and 2) during this sampling event. Likewise, *cis*-DCE concentrations in the same region were relatively low (~100 μM). Taken together, these results indicate limited bioenhancement in the source zone at 5 PV. In the transition zone (Ports 3 and 4), PCE was dechlorinated to *cis*-DCE and traces of *trans*-DCE. Interestingly, *cis*-DCE concentrations in the transition zone were higher than the expected (equilibrium) value, indicating bioenhanced dissolution from the fingered NAPL region within the transition zone. As shown in Figure 4.4A, *cis*-DCE concentrations were significantly higher in the even-numbered ports (right side of the column) than in the odd-numbered ports (left side of the column) in the transition (Ports 3 and 4) and down-gradient (Ports 5-11) zones of the column. These concentration differences likely resulted from uneven NAPL fingering on the side of the column with the even-numbered ports. The increased contact time of liquid traveling through the transition zone with greater NAPL fingering would be equivalent to increasing the source zone length. Bioenhanced dissolution from residual NAPL zones has been predicted to increase with source zone length (*39,40*), and a recent study by Glover et al. (*17*) saw greater bioenhanced dissolution from NAPL pools with transition zones than from NAPL pools with sharp boundaries (i.e., limited transition zone lengths).

Future experiments are needed to explore the influence(s) of source zone DNAPL architecture on bioenhanced dissolution.

The second side port sampling event of the mixed-NAPL column occurred after 11 PV when effluent *cis*-DCE concentrations, although still significantly higher than the expected, equilibrium concentration, were declining. As shown in Figure 4.4 B, PCE was detected in the source zone, but at concentrations below the expected, equilibrium value. TCE (150-200 μM) was also detected in Ports 1 and 2, indicating that dechlorination activity was occurring in the source zone. Both PCE and TCE were completely dechlorinated 20 cm down-gradient of the column influent (i.e., Port 4). *cis*-DCE concentrations increased throughout the source and transition zones (Ports 1-4) and indicated significant bioenhanced dissolution. In the down-gradient plume region (Ports 5-11), *cis*-DCE concentrations remained nominally stable at elevated levels (1,300 - 1,500 μM) and corresponded well to the effluent concentration. The sampling needle could not be fully inserted into the column at Ports 5 and 9 during this side port sampling event, presumably due to blockage of the side ports with individual sand grains. As a result, samples were withdrawn from the dead-volume of Port 5 and 9, likely resulting in the lower *cis*-DCE concentrations observed in these ports as compared to the rest of the plume region (see Figure 4.4B).

The final side port sampling event occurred after 17.5 PV when effluent *cis*-DCE concentrations had reached a pseudo-steady state value in the mixed-NAPL column experiment. The chlorinated ethene profile at 17.5 PV (Figure 4.4C) is comparable to the profile at 11 PV (Figure 4.4B). PCE was detected in the source zone, but unlike the previous side port profiles, was not completely dechlorinated to *cis*-DCE until about

halfway through the column. Both TCE and *cis*-DCE were detected in Ports 1-4, again indicating ongoing dechlorination activity in the source zone. *cis*-DCE concentrations increased throughout the source and transition zones (Ports 1-4) and were again at levels that indicated significant bioenhanced dissolution. In the down-gradient plume region (Ports 5-11), *cis*-DCE concentrations remained relatively stable at elevated levels (650 to 750 μM) and corresponded well with the concentration measured in the effluent. Overall, chlorinated ethene concentrations were lower at 17.5 PV as compared to 11 PV, which is consistent with the effluent data (Figure 4.3). The number of *pceA* genes could easily be quantified with the additional ~1-mL liquid samples withdrawn from the side ports during the 17.5 PV sampling event (Figures 4.4C and 4.4D). The number of *pceA* genes increased from 4.5×10^8 to 1.4×10^9 per mL over the source and transition zones (Ports 1-4), coinciding with an increase in *cis*-DCE concentrations over the same interval (see Figure 4.4C). In the down-gradient plume region of the column, the number of *pceA* genes was slightly lower than those observed in the transition zone. These results imply that *S. multivorans* was present and active in the mixed-NAPL source zone and benefited from bioenhanced NAPL dissolution. As shown in Figure 4.4D, the profiles of the *pceA* gene along the length of the column were comparable between liquid samples (i.e., from the side ports, 17.5 PV) or solid-phase (i.e., sand) samples taken after the column was destructively sampled (18.5 PV). Although the profiles are on different scales (gene copies per mL or per g sand), the matching trends indicate that liquid samples reflect biomass distribution within the column matrix.

4.3.3 NAPL Dissolution Phase of the Pure PCE-DNAPL Column Experiment

Effluent chlorinated ethene concentrations for the pure-NAPL column are shown in Figure 4.5. The dashed line in Figure 4.5 indicates the equilibrium dissolved-phase solubility of pure PCE (~1,200 μM) (*26*). In the absence of bioenhanced NAPL dissolution and assuming equilibrium mass transfer (Section 4.2.6), the total chlorinated ethene concentration (i.e., the sum of the concentrations of PCE, TCE, *cis*-DCE, and *trans*-DCE) should equal the equilibrium value. As shown in Figure 4.5, the effluent PCE concentrations were near solubility throughout the pure PCE-DNAPL column experiment. Only minimal dechlorination occurred, resulting in trace levels of TCE (< 5 μM) and *cis*-DCE (< 8 μM) in the effluent. Obviously, microbial activity did not result in enhanced contaminant dissolution in the pure PCE-DNAPL experiments. In contrast to the mixed-NAPL column experiment, the number of *pceA* genes per mL detected in the effluent sharply declined following DNAPL addition (~2 orders-of-magnitude; data not shown). Although *pceA* genes were detected at low levels throughout the experiment, the number of gene copies never exceeded those detected prior to PCE-DNAPL addition. The lack of dechlorination activity and absence of growth indicate that *S. multivorans* could not tolerate saturating concentrations of PCE, a result consistent with the observations described in Chapter 3.

4.3.4 Cumulative Mass Recoveries and Mass Transfer Enhancement Factors

Cumulative chlorinated ethene recoveries, on a molar as well as a percent basis, are summarized in Table 4.1 and Figure 4.6 for both column experiments. For a detailed description of how cumulative mass recoveries and mass transfer enhancement factors

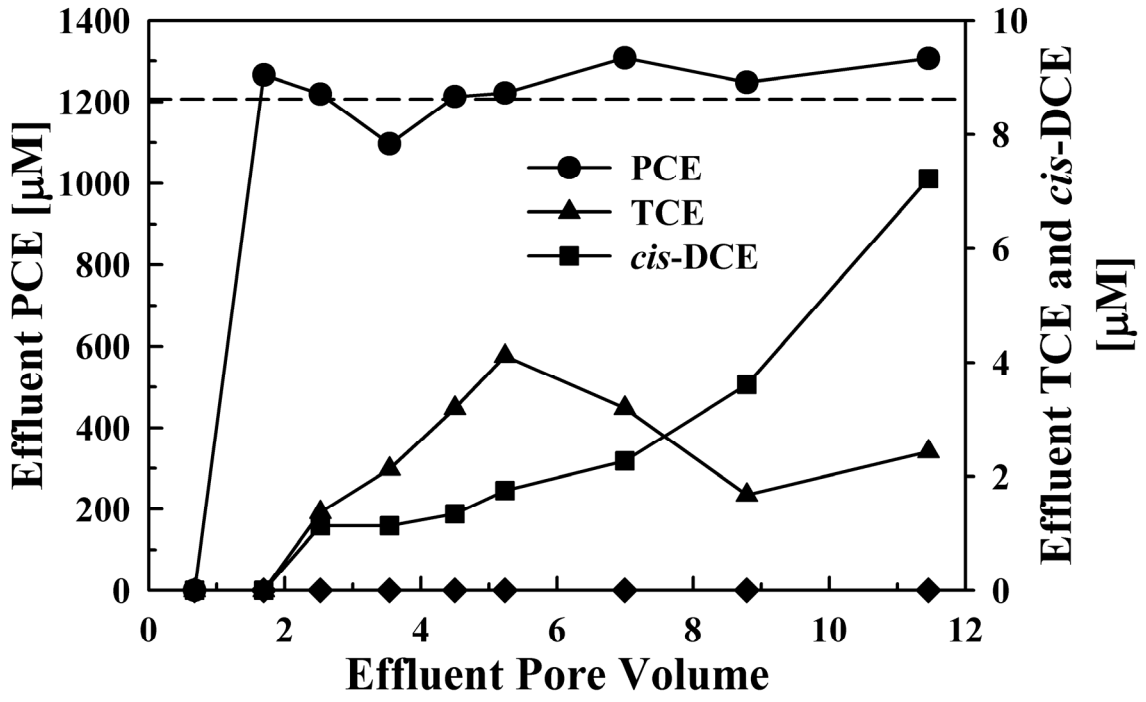

Figure 4.5 Effluent chlorinated ethene concentrations measured during the NAPL dissolution phase of the pure PCE-DNAPL column experiment. The expected chlorinated ethene concentration (1,200 μM), based on equilibrium solubility of PCE, is shown as the dashed line (see text for details).

Table 4.1 Cumulative chlorinated ethene recoveries and mass transfer enhancement factors for the mixed-NAPL and pure PCE-DNAPL column experiments.

	Column Experiment	
Parameter	**Mixed-NAPL** [a]	**PCE-DNAPL**
Initial PCE Loading (μmol)	14,231	87,008
Total Chlorinated Ethenes Recovered (μmol) [%]	7,564.4 [53.2]	5,641 [6.48]
PCE Recovered (μmol) [%]	75.8 [0.5]	5,622 [6.46]
TCE Recovered (μmol) [%]	12.5 [<0.1]	5 [<0.01]
cis-DCE Recovered (μmol) [%]	7,409.2 [52.1]	14 [0.016]
trans-DCE Recovered (μmol) [%]	66.9 [0.5]	0 [0]
Expected Abiotic PCE Recovered (μmol) [%]	1,637.6 [11.5]	5,430 [6.24]
Enhancement Factors		
Maximum Mass Transfer Enhancement Factor	13.6	1.09
Cumulative Mass Transfer Enhancement Factor	4.7	1.04

[a] 0.25/0.75 (mol/mol) PCE dissolved in hexadecane

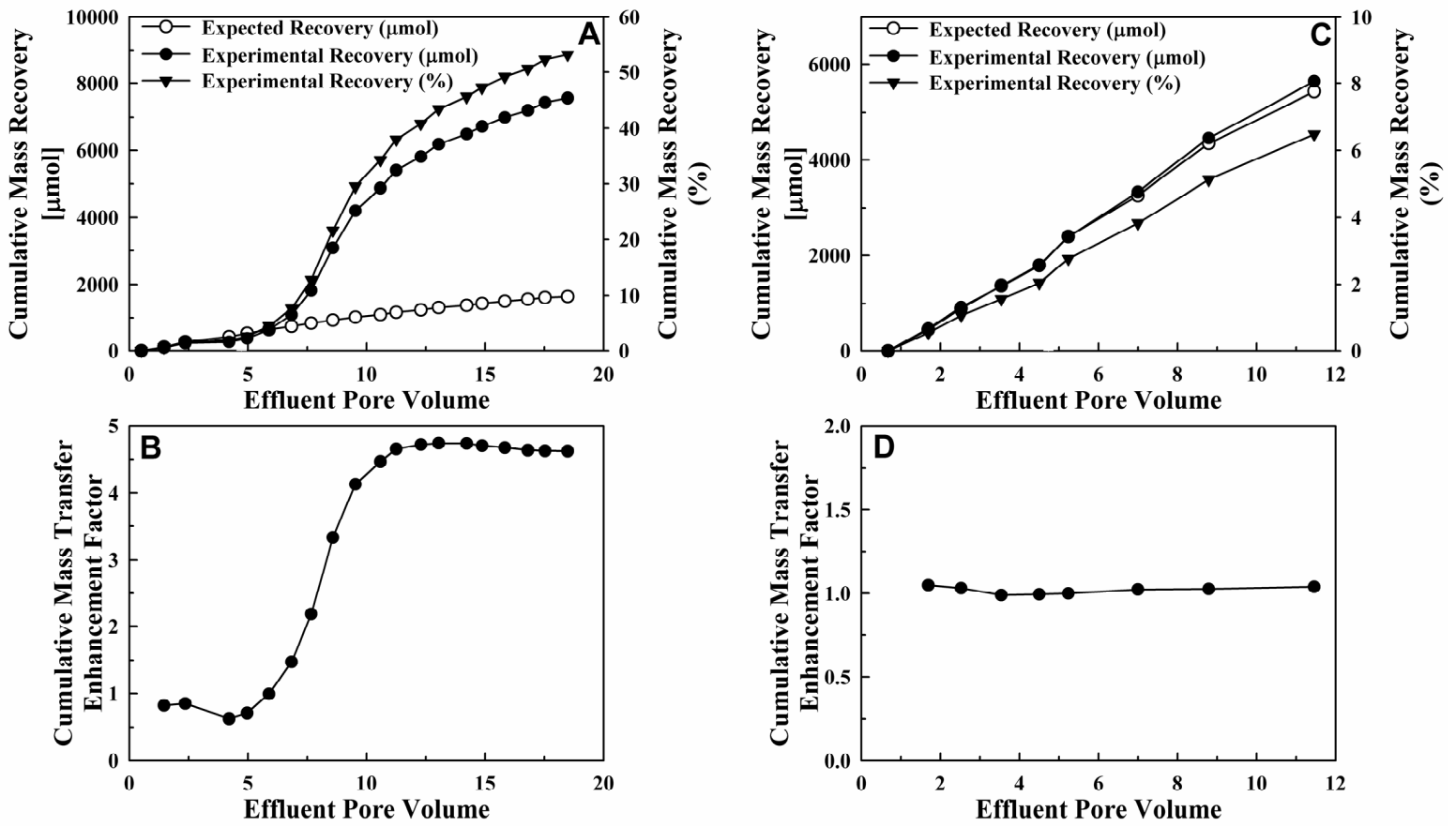

Figure 4.6 Chlorinated ethene recoveries and cumulative mass transfer enhancement factors for the mixed-NAPL and pure PCE-DNAPL column experiments: (A) cumulative mass and percent recovery for the mixed-NAPL column experiment; (B) cumulative mass transfer enhancement factor for the mixed-NAPL column experiment; (C) cumulative mass and percent recovery for the pure PCE-DNAPL column experiment; and (D) cumulative mass transfer enhancement factor for the pure PCE-DNAPL column experiment. See the text for details on how the expected (abiotic) recovery and the cumulative mass transfer enhancement factors were calculated.

were calculated, see Appendix A. For the mixed-NAPL column experiment, approximately 53% of the PCE initially present in the residual NAPL source zone was recovered in the column effluent (Figure 4.6A), primarily as *cis*-DCE (Table 4.1). This recovery was significantly greater than the expected mass recovery under abiotic conditions (11.5%; Table 4.1 and Figure 4.6A). The expected mass recovery was estimated by assuming an initial aqueous PCE solubility of ~300 μM, accounting for changing NAPL composition with PCE mass depletion (see Appendix A), and assuming equilibrium mass transfer at the operational flow rate of the column (0.25 mL/min). A cumulative mass transfer enhancement factor was calculated by dividing the experimental mass recovery by the expected mass recovery (see Appendix A for details). As shown in Figure 4.6B, the cumulative mass transfer enhancement factor was approximately unity (i.e., no enhancement) for the first 5 PV of operation of the mixed-NAPL column experiment. After 5 PV, the cumulative mass transfer enhancement rapidly increased before leveling off around 4.7. An alternative way of calculating mass transfer enhancement is by comparing experimental effluent concentrations to abiotic effluent concentrations (see Appendix A for details). Enhancement factors calculated in this manner (termed maximum herein) are the ones most commonly reported in the literature and are always higher than cumulative enhancement factors. For the mixed-NAPL column, the maximum mass transfer enhancement was 13.6-fold (Table 4.1), but this occurred at only one time during the experiment (8.6 PV; Figure 4.3). The enhancement factors determined for the mixed-NAPL column experiment, either cumulative or maximum, are comparable to the enhancement factors reported in the literature (see Chapter 2, Table 2.5) (*10-17*), which range from 1.3 to 14. In the pure PCE-DNAPL

column experiment, approximately 6.5% of the PCE initially present in the DNAPL phase was recovered in the effluent (see Table 4.1 and Figure 4.6C). This recovery was virtually identical to the expected mass recovery under abiotic conditions, assuming an equilibrium solubility of 1,200 μM. Due to the lack of significant microbial reductive dechlorination, enhancement of mass transfer was not observed in the pure PCE-DNAPL column experiment, resulting in no dissolution enhancement (i.e., cumulative and maximum enhancement factors of ~1) (Table 4.1; Figure 4.6D).

4.4 Summary and Conclusions

Microbial reductive dechlorination has emerged as a promising approach for the remediation of PCE-DNAPL source zones. Previous studies have indicated that biological activity can enhance contaminant dissolution (1.3-14 times) and that dechlorinating activity occurs in the vicinity of PCE-DNAPL source zones (*11-15,17*). Although the previous studies suggest microbial colonization of NAPL source zones, few of the studies have evaluated microbial community dynamics and composition during bioenhanced dissolution. Therefore, the activity and distribution of relevant microbial species (i.e., dechlorinators) within DNAPL source zones is poorly understood.

The goal of the continuous-flow column experiments described herein was to investigate the spatial distribution of *S. multivorans*, a PCE-to-*cis*-DCE dechlorinator, in the vicinity of a simulated PCE-NAPL source zone and to explore the relationship between microbial distribution and dissolution enhancement. The growth and distribution of *S. multivorans* was determined with a qPCR protocol that was developed

as part of this work. The qPCR protocol targeted the PCE reductive dehalogenase gene (*pceA*) of *S. multivorans*, and, to the author's knowledge, is the first qPCR protocol designed for quantitative detection of *S. multivorans*. During the mixed-NAPL experiment, qPCR analysis demonstrated a 2-3 orders-of-magnitude increase in the number of *pceA* genes detected in the column effluent following NAPL imbibition and continuous addition of electron donor (pyruvate). The growth of *S. multivorans* corresponded to the onset of PCE dechlorination and significant accumulation of *cis*-DCE. The activity of *S. multivorans* resulted in bioenhanced NAPL dissolution, characterized by a maximum enhancement factor of 13.6 and a cumulative enhancement factor of 4.7. These enhancement factors are comparable to those reported in literature (*11-15,17*). Results from both chemical (e.g., chlorinated ethene) and qPCR analyses indicated that *S. multivorans* was present and active in the mixed-NAPL source zone. Increases in cell titers (i.e., biomass) within the source zone coincided with increases in the concentrations of PCE dechlorination products (i.e., *cis*-DCE), linking the distribution and growth of *S. multivorans* with enhanced NAPL dissolution.

In contrast to the mixed-NAPL experiment, little microbial activity and no bioenhanced dissolution was observed in the pure PCE-DNAPL experiment. The lack of dechlorinating activity and the absence of microbial growth indicate that *S. multivorans* could not tolerate saturating concentrations of PCE, a result consistent with the observations described in Chapter 3. The pure PCE-DNAPL experiment represents a "worst case" scenario (i.e., the highest possible aqueous phase PCE concentrations). It is unlikely that PCE would uniformly reach such high concentrations throughout a real world DNAPL source zone due to heterogeneities in groundwater flow and DNAPL

distribution (see further discussion in Chapter 3, Section 3.5). Hence, future research should evaluate microbial distribution and bioenhanced DNAPL dissolution in more heterogeneous systems (e.g., 2-D aquifer cells), especially since the results presented herein suggest that source zone architecture (i.e., length, presence of transition zones) affects bioenhanced dissolution.

The results from the column experiments indicate several other variables that may also be critical for successful bioremediation of DNAPL source zones. These include microbial attachment and transport, potential toxic effects of high concentrations of *cis*-DCE during bioenhanced dissolution, and system buffering to maintain the pH conducive for the microbial reductive dechlorination process. At present, interactions among these variables and the key dechlorinating organisms involved in bioenhanced dissolution are poorly understood. Elucidating these interactions is likely to further promote source zone bioremediation.

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CHAPTER 5

QUANTITATIVE REAL-TIME PCR (qPCR) CORRELATES MICROBIAL ACTIVITY AND DISTRIBUTION WITH ENHANCED CONTAMINANT DISSOLUTION FROM A PCE-NAPL SOURCE ZONE: PART 2 – EXPERIMENTS WITH A PCE-TO-ETHENE DECHLORINATING CONSORTIUM

5.1 Introduction

This study is a continuation of the one-dimensional (1-D), continuous-flow column experiments described in Chapter 4. The overarching rationale for performing the additional column experiment is discussed in Section 4.1. Despite recent demonstrations of bioenhanced contaminant dissolution (*1*-*8*), the activity and distribution of relevant microbes within tetrachloroethene (PCE) dense nonaqueous phase liquid (DNAPL) source zones and the effect(s) of microbial distribution on bioenhanced dissolution are poorly understood. The column experiments described in Chapter 4 evaluated the dechlorination performance and distribution of *Sulfurospirillum multivorans*, a PCE-to-*cis*-1,2-dichloroethene (*cis*-DCE) dechlorinating isolate. The results from the initial experiments with *S. multivorans* demonstrated microbial activity within a simulated PCE-NAPL source zone and provided a link between contaminant

dissolution and microbial growth. Although the activity of *S. multivorans* resulted in bioenhanced NAPL dissolution (4.7-fold, cumulatively), this isolate is only capable of dechlorinating PCE to *cis*-DCE, a toxic intermediate in the microbial reductive dechlorination pathway. In the published reports of bioenhanced dissolution, dechlorination also stalled at *cis*-DCE and/or VC, despite the use of microbial consortia capable of complete PCE detoxification (i.e., ethene formation) (*1-8*). Dechlorination of PCE to *cis*-DCE within NAPL source zones may be beneficial in reducing source zone longevity and increasing the bioavailability of dechlorination products (e.g., *cis*-DCE) to down gradient processes acting on dissolved-phase contaminants. Nevertheless, accumulation of toxic intermediates during source zone bioremediation is undesirable.

Efficient and complete dechlorination of PCE to ethene depends on the presence of multiple dechlorinating organisms (*9*). Several microbial isolates transform PCE to *cis*-DCE (e.g., *Sulfurospirillum* spp. (*10,11*), *Geobacter lovleyi* strain SZ (*12*), and *Dehalobacter* spp. (*13,14*)). Only members of the *Dehalococcoides* group have been shown to dechlorinate beyond *cis*-DCE to vinyl chloride (VC) and benign ethene (summarized in (*9*)). None of the *Dehalococcoides* isolates grow with all chlorinated ethenes, and VC dechlorination is a slow, cometabolic process (i.e., growth does not occur) in certain *Dehalococcoides* strains (see Chapter 2, Table 2.4). At present, the contributions of individual dechlorinating organisms to bioenhanced dissolution and the factor(s) limiting the ability of *Dehalococcoides* spp. to produce significant quantities of ethene in source zones are currently unknown.

These knowledge gaps were addressed in a 1-D, continuous-flow column experiment. The column contained a mixed-NAPL source zone (0.25/0.75 mol/mol PCE

dissolved in hexadecane) and was inoculated with a robust, PCE-to-ethene dechlorinating consortium, BDI-SZ, selected because: (i) it contained multiple *Dehalococcoides* strains and two PCE-to-*cis*-DCE dechlorinating populations (*Geobacter lovleyi* strain SZ and a *Dehalobacter* species); (ii) dechlorinating consortia are often used as bioaugmentation inocula (*15,16*); and (iii) the microbial composition of the selected dechlorinating consortium is similar to commercially available bioaugmentation inocula (e.g., KB-1 [SiREM, http://www.siremlab.com]; SDC-9 [Shaw, http://www.shawgrp.com]).

5.2 Materials and Methods

The column experiment with BDI-SZ, a PCE-to-ethene dechlorinating consortium, was performed as described for the *S. multivorans* column experiments (Chapter 4, Section 4.2). This section only describes modifications and additions to the procedures outlined in Chapter 4.

5.2.1 Chemicals

In addition to the chemicals used in the previous column experiments (Chapter 4, Section 4.2.1), gaseous VC (≥99.5%; Fluka Chemical Corp., Ronkonkoma, New York) and ethene (99.5%; Scott Specialty Gases, Durham, North Carolina) were also utilized.

5.2.2 NAPL Preparation

Based on the results presented in Chapter 4, the column experiment was performed with a mixed-NAPL (0.25/0.75 mol/mol PCE dissolved in hexadecane [HD])

source zone. Use of the mixed-NAPL reduced the equilibrium dissolved-phase PCE concentrations to below those inhibitory for many dechlorinating organisms (Chapter 3). The anoxic PCE/HD mixed NAPL was prepared as described in Chapter 4, Section 4.2.2. The equilibrium solubility of PCE in the mixed-NAPL was ~300 μM (50 mg/L).

5.2.3 Medium Preparation and Column Inoculum

Reduced, anaerobic, mineral salts medium was prepared as described in Chapter 4, Section 4.2.3, except for the following modifications: the concentration of $Na_2S \cdot 9H_2O$ was reduced to 0.2 mM and the concentration of anhydrous L-cysteine hydrochloride was increased to 1 mM. Lactate, as opposed to pyruvate, was added as electron donor at a final concentration of 20 mM (where indicated). Lactate was provided in excess so that electron donor limitations did not control microbial activity and bioenhanced dissolution.

The PCE-to-ethene dechlorinating culture used as inoculum for the column experiment was developed by mixing cultures of the PCE-to-*cis*-DCE dechlorinating isolate, *Geobacter lovleyi* strain SZ (DSM 17278, (*12*)), with a PCE-to-ethene dechlorinating consortium, Bio-Dechlor INOCULUM (BDI). BDI has been successfully used in bioaugmentation field applications (*17*) and contains at least three *Dehalococcoides* strains (strains FL2, GT, and BAV1 (*16*)) and a PCE-to-*cis*-DCE dechlorinating *Dehalobacter* species (*18*). *G. lovleyi* strain SZ was added to BDI since strain SZ-like organisms are widely distributed in the environment and present at many contaminanted sites (Chapter 7 and references (*19-21*)). In addition, a strain SZ-like population is present in the bioaugmentation inoculum KB-1, which has been used

successfully at a number of sites (*15,22*) and is commercially available (SiREM; http://www.siremlab.com). Recent studies have demonstrated growth of SZ-like organisms in the KB-1 consortium during PCE and TCE dechlorination to *cis*-DCE (*21*). The development of the PCE-to-ethene dechlorinating consortium, subsequently referred to as BDI-SZ, allowed for simultaneous assessment of multiple, environmentally-relevant dechlorinating populations during bioenhanced dissolution.

The BDI-SZ consortium received periodic additions of PCE (0.33 mM) as electron acceptor and lactate (20 mM) as electron donor. The dechlorination performance of BDI-SZ was stable over a period of several months before initiation of the column experiment. Before the column was packed (see Section 5.2.4), samples of the BDI-SZ consortium were collected for DNA extraction and quantitative real-time PCR (qPCR) analysis.

5.2.4 Column Operation

This section describes the operation of the 1-D column experiment, which occurred in multiple stages. A schematic diagram of the 1-D column system is shown in Chapter 4, Figure 4.1. The column was packed as described in Chapter 4 (Section 4.2.5.1) with Federal Fine Ottawa sand and a suspension of the BDI-SZ consortium as the resident aqueous phase. The pore volume (PV) of the column was estimated from mass differences to be ~407 mL, which is comparable to the PV of the two experiments with *S. multivorans* described in Chapter 4. After packing was completed, the column remained in the glove bag system for ~24 hours before reduced medium (without lactate) was continuously flushed through the column at 0.25 mL/min for 3.3 PV. To monitor the

elution (i.e., washout) of the added microorganisms (microbial elution phase), effluent samples (10-20 mL) were taken periodically for DNA extraction and qPCR analysis. The medium did not contain a carbon source or electron donor (i.e., lactate) during this phase, nor did the column yet contain PCE-NAPL; therefore, minimal growth and/or decay of the microbial organisms were assumed during the microbial elution phase. Following the microbial elution phase, the column was imbibed with the anoxic mixed-NAPL as described in Chapter 4 (Section 4.2.5.3). At the conclusion of the imbibition process, the 10 cm (nominal) source zone contained approximately 16 mL of residual (entrapped) NAPL ganglia, corresponding to an organic phase saturation of approximately 0.24 over the first 10 cm of the column. Although the NAPL distribution within the source zone was relatively uniform, some fingering of the NAPL was apparent. The fingering was not factored into the determination of residual saturation, which may have resulted in an overestimation of source zone NAPL saturation. Ports 1 and 2 were within the source zone, and Ports 4-11 were in the plume region. Due to some fingering of the imbibed NAPL, Port 3 was in a transition zone between the source zone and the plume region.

After the residual NAPL source zone was established in the column, effluent and side port samples were periodically taken to monitor bioenhanced dissolution (NAPL dissolution phase). The column was continuously operated and dechlorination activity monitored for approximately 3 months after NAPL imbibition. During this time, the influent contained 20 mM lactate. The influent system was initially operated at 0.25 mL/min (hydraulic retention [residence] time of approximately 1.1 days). At 21.6 PV of flushing, the flow was reduced to 0.1 mL/min (residence time of approximately 2.8 days) for the remainder of the experiment. An unintentional flow interruption occurred for

approximately 22 hours at 16.8 PV. Effluent samples (~20 mL) were collected every 1-3 days and divided into separate sub-samples for chlorinated ethene, pH, and microbial analyses. The side ports along the length of the column were also sampled periodically for chlorinated ethenes and/or microbial analysis. For further information on sampling procedures, see Chapter 4, Section 4.2.5.4. After depletion of PCE from the mixed-NAPL and a decline in chlorinated ethene effluent concentrations to < 5 μM, a ~1.1 pore volume pulse (451 mL) of VC-amended medium was injected into the column between 32.8 and 33.9 PV of column operation to assess whether conditions within the column were favorable for ethene production. During injection of the pulse, aqueous phase VC concentrations within the influent were periodically monitored and decreased from 97.5 μM to 62.5 μM during the pulse. The decrease in aqueous phase VC concentrations in the influent is likely a result of the changing ratio between the headspace and aqueous phase volumes within the influent reservoir. The ratio between the headspace and aqueous phase volumes changed since medium was continuously withdrawn from the reservoir. VC is a volatile compound that partitions between the aqueous phase and headspace; therefore, a larger headspace volume would result in reduced aqueous phase VC concentrations. Alternatively, VC sorption to the influent medium reservoir septum may have occurred and resulted in the reduced aqueous phase VC concentrations. Based on measured aqueous phase VC concentrations in the influent, approximately 34.6 μmol of VC was injected into the column during this pulse. VC elution and/or ethene production were monitored in effluent samples for the duration of the experiment to estimate recovery of the pulse as VC and/or ethene. At the conclusion of the column

experiment, the column was destructively sampled as described in Chapter 4, Section 4.2.5.5.

5.2.5 DNA Extraction and Quantitative Real-Time PCR (qPCR) Analysis

DNA was extracted from the aqueous and solid-phase (sand) samples as described in Chapter 4, Section 4.2.7. qPCR analysis quantified the number of 16S rRNA genes of *Dehalococcoides* spp., *Dehalobacter* spp., and *Geobacter lovleyi* strain SZ. qPCR analysis was performed with an ABI 7500 Fast Real-Time PCR System (Applied Biosystems [ABI], Foster, California) using the standard 7500 operating mode. TaqMan-based qPCR analysis was used to quantify the number of *Dehalococcoides* 16S rRNA gene copies as described (*16*). Each well of a MicroAmp Optical 96-Well Reaction Plate (ABI) contained 1x TaqMan universal PCR master mix (ABI), 300 nM probe, 300 nM of each primer, and 3 µL of template DNA in a total reaction volume of 30 µL. The PCR temperature program was as follows: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 58°C. Standard curves were generated for the *Dehalococcoides* 16S rRNA gene as described (*16*). For *Dehalococcoides*, the terms "16S rRNA gene copies" and "cell numbers" are per mL or per gram of sample and are used interchangeably because the known *Dehalococcoides* organisms contain a single 16S rRNA gene copy per genome (*23,24*).

qPCR analysis to quantify *Dehalobacter* 16S rRNA genes was performed using SYBR Green-based detection chemistry as described by Smits et al. (*25*) and Daprato et al. (*26*). The qPCR reaction mixture contained 15 µL of Power SYBR Green PCR master mix (ABI), 300 nM of each primer, and 3 µL of template DNA in a total reaction volume

of 30 μL. The PCR temperature program was as follows: 2 min at 50°C, 15 min at 95°C followed by 40 cycles of 15 sec at 94°C, 30 sec at 58°C, and 30 sec at 72°C. Standard curves were generated following the procedure outlined in Ritalahti et al. (*16*), and used a 10-fold dilution series of quantified plasmid DNA (concentrations determined spectrophotometrically at 260 nM). Each plasmid carried a single copy of the 16S rRNA gene of *Dehalobacter restrictus* (DSM 9455). The number of 16S rRNA genes in the genome of *Dehalobacter restrictus* has not been determined; the cell number estimates reported herein assume one 16S rRNA gene copy per *Dehalobacter* cell and are reported per mL or per gram of sample.

qPCR analysis to quantify *Geobacter lovleyi* strain SZ was performed using SYBR Green-based detection chemistry and primers that targeted the 16S rRNA gene. Primer Geo73F (5'-CTTGCTCTTTCATTTAGTGG -3') was designed by Duhamel and Edwards (*19*), while Geo196R (5'-GAATCAGGAGCATATTC-3') is the reverse complement of primer Geo196F, which was designed as part of the work described in Chapter 7. The specificity of the primer pair was experimentally confirmed with DNA from close relatives (*G. thiogenes*, *G. sulfurreducens*, and *G. metallireducens*) and selected reductively dechlorinating species (*Desulfuromonas michiganensis* strain BB1, *Anaeromyxobacter dehalogenans* strain 2CP-C, *Sulfurospirillum multivorans*, *Dehalobacter restrictus*, *Dehalococcoides* sp. strain BAV1, *Desulfitobacterium* sp. strain Viet1, and culture "*Clostridium bifermentans* strain DPH-1"). The Geo73F/Geo196R primer pair amplified DNA from both *G. lovleyi* strain SZ and *G. thiogenes*, but did not amplify DNA from any of the other organisms. No amplification was observed with DNA extracted from the BDI consortium (before addition of strain SZ and development

of BDI-SZ), indicating that the selected primer pair, although unable to distinguish strain SZ and *G. thiogenes*, only monitored the growth and distribution of strain SZ in samples from the column experiment. The qPCR reaction mixture contained 15 µL of Power SYBR Green PCR master mix (Applied Biosystems), 300 nM of each primer, and 3 µL of template DNA in a total reaction volume of 30 µL. The PCR temperature program was as follows: 2 min at 50°C, 15 min at 95°C followed by 40 cycles of 30 sec at 94°C, 30 sec at 51°C, and 30 sec at 72°C. Standard curves were generated following the procedure outlined by Ritalahti et al. (*16*), and used a 10-fold dilution series of quantified plasmid (concentration determined spectrophotometrically at 260 nM) carrying a single copy of the 16S rRNA gene of *Geobacter lovleyi* strain SZ. The genome of strain SZ contains two 16S rRNA gene copies (www.jgi.doe.gov); therefore, dividing gene copy numbers by a factor of two yielded the cell numbers, which are reported per mL or per gram of sample.

5.2.6 Analytical Methods

Aqueous phase (1 mL) samples were collected and analyzed by gas chromatography for chlorinated ethenes and ethene as described in Chapter 4 (Section 4.2.9). Standard calibration curves for chlorinated ethenes and ethene were prepared as described (*27-29*). The effluent pH was periodically determined as described in Chapter 4 (Section 4.2.9).

5.3 Results and Discussion

5.3.1 Microbial Elution Phase

When the column experiment was initiated, the BDI-SZ consortium used for packing the column (i.e., the inoculum) contained 3.5 ± 1.1 x 10^5 *Dehalobacter* cells per mL, 6.3 ± 0.9 x 10^7 *Geobacter* cells per mL, and 1.8 ± 0.3 x 10^8 *Dehalococcoides* cells per mL. Taking into account the column pore volume (407 mL) and these cell estimates, 1.4 ± 0.4 x 10^8 *Dehalobacter* cells, 2.6 ± 0.4 × 10^{10} *Geobacter* cells, and 7.5 ± 1 × 10^{10} *Dehalococcoides* cells were initially added to the entire column. After packing, the column remained in the N_2-flushed glove bag system for approximately 24 hours. After this period without flow, reduced medium (without lactate) was continuously pumped through the column for 3.3 PV and elution (i.e., washout) of the target microorganisms was monitored.

The number of cells per mL eluted from the column and recovered in the effluent during the microbial elution phase is shown in Figure 5.1. For *Dehalobacter*, a significant decrease (~2 orders-of-magnitude) was observed in the number of cells per mL between the inoculum (3.5 ± 1.1 x 10^5 cells per mL) and the first effluent sample (5.3 ± 0.3 x 10^3 cells per mL; see Figure 5.1A). For *Geobacter* and *Dehalococcoides*, the number of cells per mL in the effluent (1.7 ± 0.8 x 10^7 and 1.3 ± 0.1 x 10^8, respectively) matched relatively closely to those measured in the inoculum (6.3 ± 0.9 x 10^7 and 1.8 ± 0.3 x 10^8, respectively). For all three dechlorinators, effluent cell numbers remained relatively constant over the first 1.5 PV, after which the effluent concentrations significantly declined (Figure 5.1A). For *Dehalobacter*, the effluent cell numbers at the

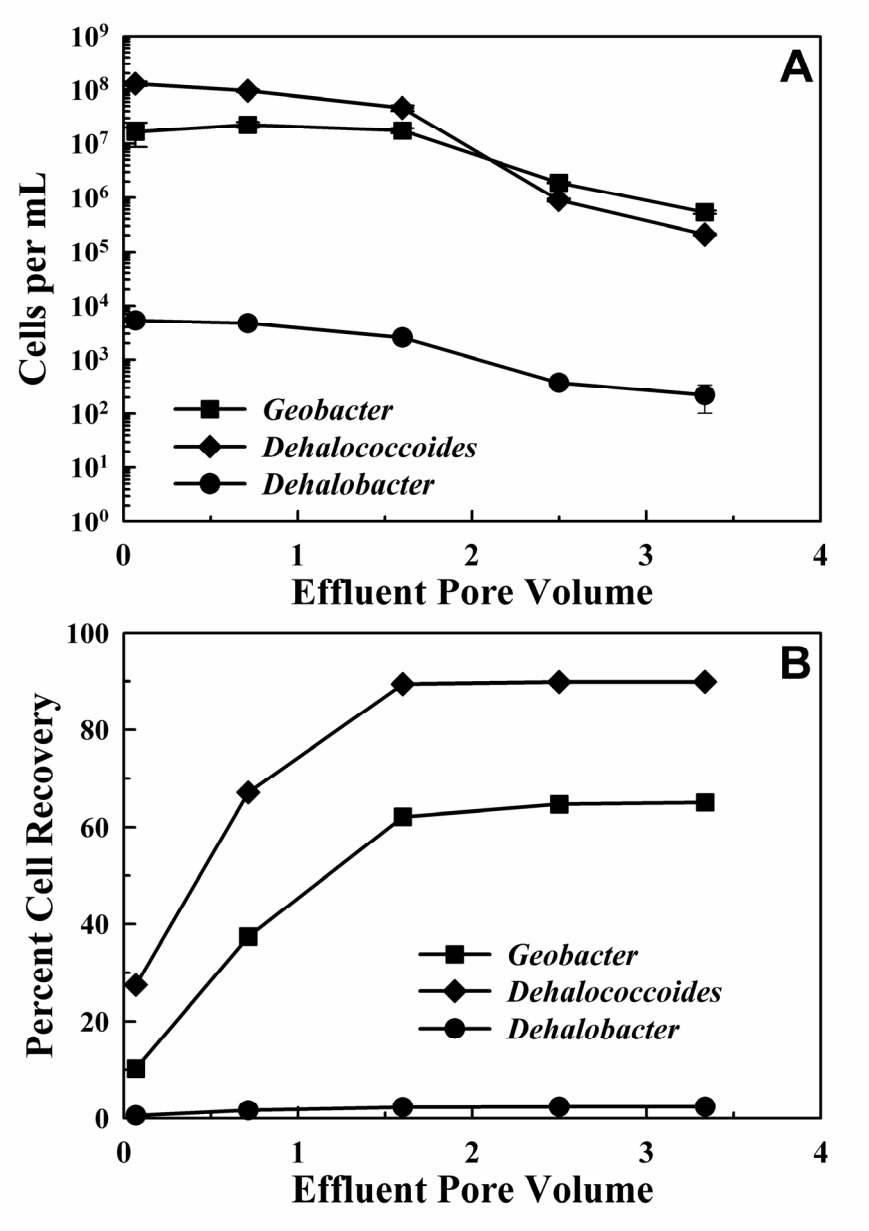

Figure 5.1 Effluent cell numbers per mL (A) and percent cell recoveries (B) for *Geobacter*, *Dehalococcoides*, and *Dehalobacter* during the microbial elution phase.

end of the microbial elution phase approached the quantification limit of the *Dehalobacter*-targeted qPCR approach (1.3×10^2 cells per mL).

Cumulative cell recoveries were determined by taking the area under the curve in Figure 5.1A, while percent recoveries compared the cumulative cell recoveries to the total numbers of cells added to the column (see above). Calculation of percent recoveries assumed no growth or decay of each dechlorinating population, which is reasonable since the influent medium did not contain a carbon source or electron donor during this experimental phase and the column did not yet contain PCE-NAPL. As shown in Figure 5.1B, the percent of cells added to the column and recovered in the effluent spanned from 2% (*Dehalobacter*) to 90% (*Dehalococcoides*) during the 3.3 PV microbial elution phase. In the previous column experiments, 25-30% of *S. multivorans* cells added to the columns were recovered (see Chapter 4). Taken together, these results suggest organism specific attachment/retention characteristics; further exploration of microbial attachment and transport processes is needed. Interestingly, *Dehalobacter* cell numbers were below detection throughout the rest of the experiment (in both effluent and side port samples). *Dehalobacter* cells were also not detected in the solid-phase (sand) samples following the completion of the experiment and sectioning of the column, indicating that the added *Dehalobacter* organisms were not attached to the porous medium (sand). These data suggest that the *Dehalobacter* cells added to the column lysed during the initial setup (packing) and the subsequent 24-hour, no-flow condition. This hypothesis is consistent with the 98% difference observed between the number of *Dehalobacter* cells per mL in the inoculum and the first effluent sample (see above), a difference that was not observed for *Geobacter* and *Dehalococcoides*. Additional studies are needed to evaluate the

survival (resistance) and resilience of *Dehalobacter* spp. during bioaugmentation; such information has practical relevance for bioremediation field applications and may play a significant role in the performances of bioaugmentation inocula.

5.3.2 NAPL Dissolution Phase

5.3.2.1 Effluent Chlorinated Ethene and Ethene Concentrations. Effluent chlorinated ethene and ethene concentrations during the NAPL dissolution phase are shown in Figure 5.2A. The initial expected (abiotic) effluent chlorinated ethene concentration, assumed to be the equilibrium solubility of PCE in the mixed NAPL, was ~300 μM (black, dashed line in Figure 5.2). The gradual decline in the expected (abiotic) effluent chlorinated ethene concentration (black, dashed line in Figure 5.2) was estimated by accounting for changing NAPL composition with PCE mass depletion (i.e., PCE mass depletion effectively lowered the PCE mole fraction in the mixed-NAPL; see Appendix A for more information). In the absence of bioenhanced NAPL dissolution and assuming equilibrium mass transfer (see Chapter 4, Section 4.2.6), the total chlorinated ethene concentration (i.e., the sum of the concentrations of PCE, TCE, *cis*-DCE, *trans*-DCE, VC, and ethene) should equal the equilibrium value.

During the first 4 PV of the NAPL dissolution phase, minimal dechlorination of PCE occurred (Figure 5.2), although TCE (~ 40 μM), *cis*-DCE (~20 μM), and VC (< 5 μM) were detected in the effluent samples. Between 4 PV and 10.5 PV, PCE was completely dechlorinated to *cis*-DCE and small amounts of VC (4 – 25 μM). The total concentration of chlorinated ethenes (primarily PCE and *cis*-DCE) remained close to the expected (abiotic) effluent chlorinated ethene concentration for approximately the first 11

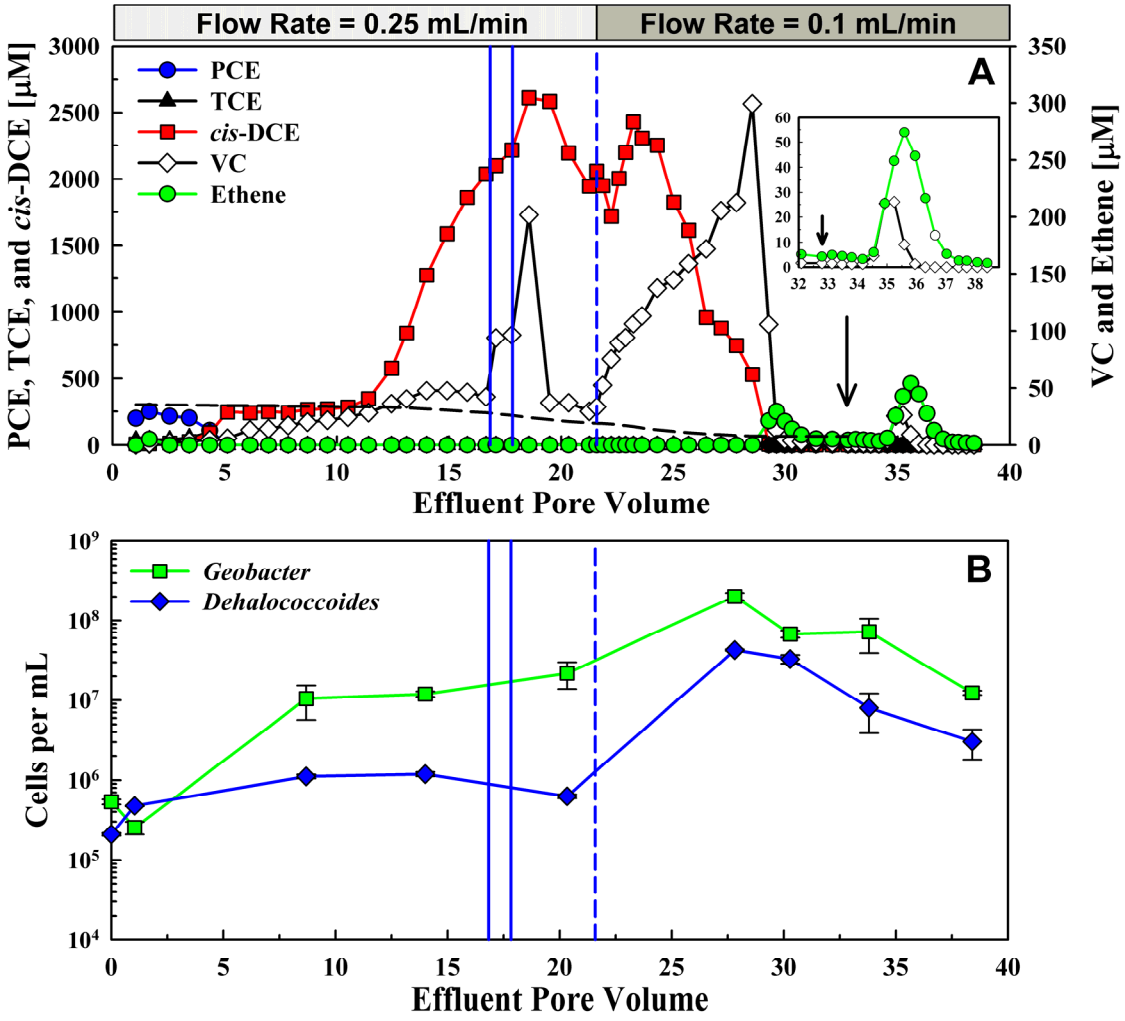

Figure 5.2 Effluent chlorinated ethene concentrations (A) and effluent cell numbers per mL (B) measured during the NAPL dissolution phase of the column experiment. In (A), the expected chlorinated ethene concentration based on equilibrium solubility of PCE is shown with the black dashed line (left-had-side y-axis); the expected value was initially ~300 µM but decreased with changing NAPL composition due to PCE mass depletion (see text for details). The inset in (A) shows effluent VC and ethene concentrations after a ~1.1 PV pulse of VC-amended medium was flushed through the column (the pulse began at 32.8 PV; see arrows). In both (A) and (B), the pore volume residing in the column during the flow interruption (~22 hours at 16.8 PV) is bracketed by the solid blue lines, and the change in flow rate from 0.25 mL/min to 0.1 mL/min is demarcated by the dashed blue line (21.6 PV).

PV, indicating that biologically-enhanced dissolution of PCE from the NAPL did not occur during the initial stage of column operation. Side port samples collected at 8.7 PV indicated minimal dechlorination activity in the source zone but complete conversion of PCE to *cis*-DCE down gradient of the source zone (data not shown), corroborating that significant biological activity within a source zone is required for bioenhanced dissolution. After 11 PV of operation, effluent *cis*-DCE concentrations increased to as high as ~2,600 μM (see Figure 5.2); this concentration is significantly higher than the expected (abiotic) chlorinated ethene concentration, indicating bioenhanced dissolution of PCE from the NAPL phase. *cis*-DCE production and bioenhancement gradually declined after 19.5 PV. When the flow rate was decreased at 21.6 PV, *cis*-DCE concentrations initially rebounded to ~2,400 μM before continuing to fall below 5 μM by 30 PV. The increase in *cis*-DCE concentrations after the flow rate change likely resulted from the increased residence time.

Effluent VC concentrations reached a pseudo-steady state level of 40-50 μM at the initial flow rate of 0.25 mL/min (Figure 5.2). A spike in VC concentration to ~200 μM was observed following the flow interruption at 16.8 PV; however, VC concentrations returned to the pseudo-steady state level when flow resumed. After the flow rate was reduced to 0.1 mL/min, VC concentrations steadily increased to a maximum of ~300 μM (28.5 PV, Figure 5.2). Taken together, these results suggest that the column residence time (1.1 days at 0.25 ml/min; 2.8 days at 0.1 ml/min) was insufficient to achieve significant dechlorination beyond *cis*-DCE.

The sharp decrease in the total chlorinated ethene concentration (primarily *cis*-DCE and VC) by 30 PV indicated complete depletion of PCE from the mixed-NAPL.

Interestingly, the decline in *cis*-DCE and VC concentrations corresponded to ethene production (see Figure 5.2). In fact, ethene (21 μM) was first detected at 29.3 PV when *cis*-DCE and VC dropped from 530 μM and 300 μM, respectively, in the previous sample (28.5 PV) to 6 μM and 105 μM, respectively, by 29.3 PV. These results suggest that VC dechlorination to ethene was inhibited by the presence of elevated concentrations of *cis*-DCE, as has been previously documented for other dechlorinating consortia (*30,31*). Accumulation of *cis*-DCE, therefore, might limit complete detoxification of PCE to ethene unless system residence times are sufficiently long for sequential conversion of *cis*-DCE to VC before VC conversion to ethene within the column. Future work is needed to address the influence of residence times and *cis*-DCE toxicity on complete detoxification of PCE during bioenhanced dissolution. To assess whether conditions within the column were conducive to VC dechlorination and ethene production, the influent medium was amended with VC and effluent samples were taken to monitor for VC elution and/or ethene production. The results of the VC pulse through the column are presented and discussed in Section 5.3.4.

5.3.2.2 Effluent pH. Periodic pH measurements were taken during the NAPL dissolution phase (data not shown). While the pH trends were not as clear as those demonstrated for the *S. multivorans* mixed-NAPL column (see Chapter 4, Figure 4.3), increasing concentrations of PCE dechlorination products generally correlated to decreases in effluent pH. As described in Chapter 4, Section 4.3.2.1, the pH decrease was attributed to the production of hydrochloric acid during each reductive dechlorination step. Even though the influent medium was buffered (60 mM $NaHCO_3$), effluent pH decreased from

7.25 to a minimum of 6.77. This pH reduction is comparable to that observed in the *S. multivorans* mixed-NAPL column experiment. pH levels below the optimum levels of dechlorinators (e.g., 7.2-7.4 for *Dehalococcoides* (*32,33*)) may have contributed to the lack of VC and ethene production (see Chapter 4, Section 4.3.2.1).

5.3.2.3 Effluent Cell Numbers. *Geobacter* and *Dehalococcoides* cell numbers in effluent samples taken during the NAPL dissolution phase are shown in Figure 5.2B. Discussion of microbial growth and distribution is limited to *Geobacter* and *Dehalococcoides* organisms since *Dehalobacter* cell numbers were below detection throughout the NAPL dissolution phase (see Section 5.3.1). Growth of both the *Geobacter* and *Dehalococcoides* populations was observed during the NAPL dissolution phase (Figure 5.2B). For example, the number of *Geobacter* and *Dehalococcoides* cells increased from $5.4 \pm 0.4 \times 10^5$ cells per mL and $2.1 \pm 0.1 \times 10^5$ cells per mL, respectively, directly after NAPL imbibition and lactate amendment to $1.1 \pm 0.5 \times 10^7$ per mL and $1.1 \pm 0.1 \times 10^6$ per mL, respectively, at 8.7 PV (Figure 5.2). The number of *Geobacter* ($\sim 10^7$) and *Dehalococcoides* ($\sim 10^5$-10^6) cells detected in the effluent remained relatively constant over the first 21 PV of column operation. In general, *Geobacter* cell numbers were more than one order-of-magnitude higher than *Dehalococcoides* cell numbers during this period, which is consist with the production of substantial quantities of *cis*-DCE, limited production of VC, and no ethene formation over the same period (Figure 5.2A). Although cell numbers of both organisms increased after the flow rate was reduced, *Dehalococcoides* cell numbers increased by approximately 2 orders-of-magnitude while *Geobacter* cell numbers only increased by approximately one order-of-magnitude (Figure

5.2B). The relative changes in *Dehalococcoides* and *Geobacter* cell numbers are consistent with decreased *cis*-DCE production and increased VC production between 21 PV and 30 PV (Figure 5.2A). After reaching maxima at 27.8 PV, the number of *Geobacter* and *Dehalococcoides* cells declined throughout the remainder of the experiment, which coincided with the sharp decrease in the total chlorinated ethene concentration due to depletion of PCE from the mixed-NAPL.

5.3.2.4 Side Port Samples. During the NAPL dissolution phase, the side ports along the length of the column were sampled at 8.7, 14, 20.3, 27.8, and 30.3 PV of continuous flushing. The results from the side ports support the conclusions drawn from the effluent data (Figure 5.2) and confirm that both *Geobacter* and *Dehalococcoides* cells were present and active in the mixed-NAPL source zone. The results from all of the side port sampling events are generally consistent with one another; therefore, only representative results are presented here.

The results from the side port samples taken at 14 PV are shown in Figure 5.3, where Port 1 is nearest the influent and Port 11 is nearest the effluent. Ports 1 and 2 were within the source zone, and Ports 4-11 were in the plume region down gradient of the source zone. Due to some fingering of the imbibed NAPL, Port 3 was in a transition zone between the source zone and the plume region. The data presented in Figure 5.3A indicate significant dechlorination activity in the source zone. Although PCE was detected at levels close to the expected concentration (269 μM; dashed line in Figure 5.3A) in the source zone, TCE (100 μM) and *cis*-DCE (1,500 μM) were also observed. The high levels of *cis*-DCE within the source zone and adjacent ports show that bioenhanced dissolution was occurring, a result that is consistent with the effluent data at

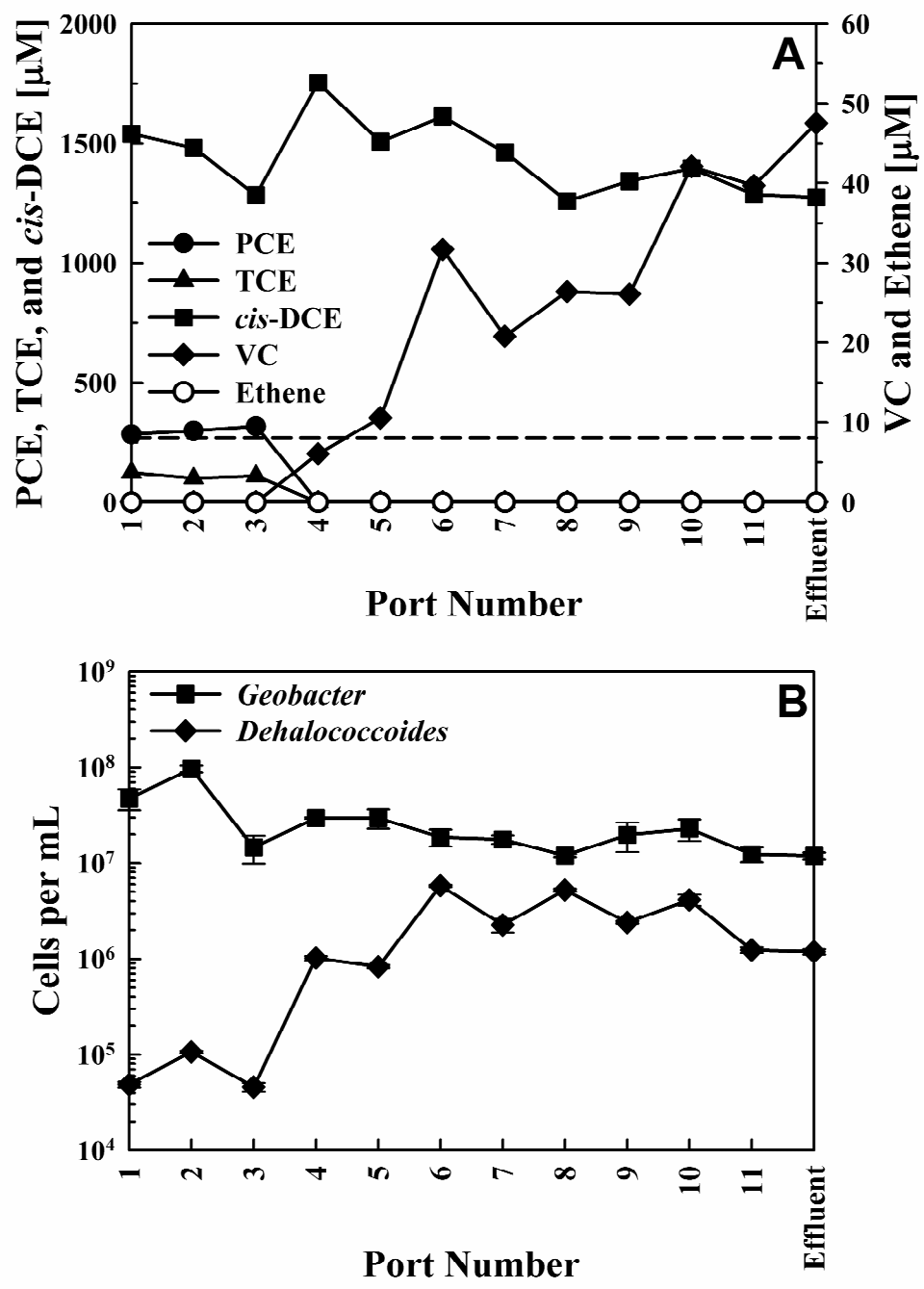

Figure 5.3 Side port chlorinated ethene concentrations (A) and cell numbers per mL for *Geobacter* and *Dehalococcoides* (B) following 14 pore volumes of flushing. Port 1 is nearest the influent and in the source zone while Port 11 is nearest the effluent. Effluent concentrations taken at the same time are shown for comparison. The expected chlorinated ethene concentration (269 µM) at 14 PV, based on equilibrium solubility of PCE and accounting for changing NAPL composition due to PCE mass depletion (see text for details), is shown with the dashed line in (A).

14 PV (see Figure 5.2A). VC was not detected in the mixed-NAPL source zone (Ports 1-3). Down gradient of the source zone, PCE and TCE were completely dechlorinated, *cis*-DCE concentrations remained relatively stable, and VC concentrations increased (Ports 4-11). VC was produced at significantly lower levels than *cis*-DCE (Figure 5.3A). Ethene was not observed in any sample from the 14 PV side port sampling event (Figure 5.3A). As shown in Figure 5.3B, the distribution of *Geobacter* and *Dehalococcoides* generally correlated with the chlorinated ethene concentration profile. For example, the number of *Geobacter* cells detected in the source zone (4.7×10^7 to 9.7×10^7 per mL) was slightly higher than the number detected in the plume region (1.2×10^7 to 2.8×10^7 per mL), consistent with complete conversion of PCE to *cis*-DCE by Port 4. In contrast, *Dehalococcoides* cell numbers were significantly lower in the source zone (4.6×10^4 to 1.1×10^5 per mL) than in the plume region (8.3×10^5 to 5.8×10^6 per mL), consistent with VC production only occurring down gradient of the source zone.

Although VC was not produced in the source zone at the 14 PV side port sampling event, VC was detected at trace levels (<10 μM) within the source zone at later times. For example, Figure 5.4 shows that VC was produced in the source zone (Ports 1 and 2) at the 27.8 PV sampling event. *Dehalococcoides* cell numbers within the source zone at 27.8 PV (Figure 5.4) were approximately an order-of-magnitude greater than those observed at 14 PV (Figure 5.3B). These results indicate that *Dehalococcoides* organisms successfully colonized and were active within a PCE-NAPL source zone. Interestingly, *Dehalococcoides* cell numbers generally increased 1 to 2 orders-of-magnitude over the length of the column (e.g., from the source zone area to the column exit) (Figures 5.3B and 5.4). In fact, the increase in *Dehalococcoides* cell numbers

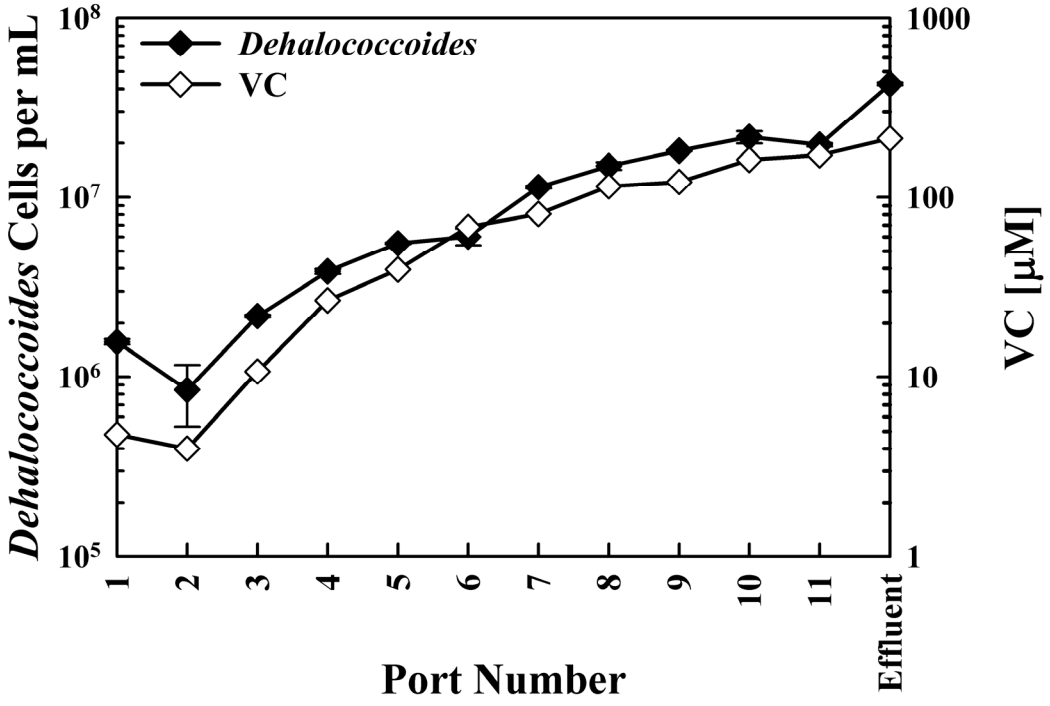

Figure 5.4 Side port profiles for VC and *Dehalococcoides* following 27.8 pore volumes of flushing. Port 1 is nearest the influent and in the source zone while Port 11 is nearest the effluent. Effluent concentrations taken at the same time are shown for comparison.

throughout the column often directly correlated to parallel increases in VC concentrations, as illustrated in the side port samples from 27.8 PV (Figure 5.4).

5.3.3 Cumulative Mass Recoveries and Mass Transfer Enhancement Factors

Cumulative chlorinated ethene recoveries, on a molar as well as a percent basis, are summarized in Table 5.1 and Figure 5.5A. A detailed description of how cumulative mass recoveries and mass transfer enhancement factors were calculated is provided in Appendix A. Cumulative mass recoveries include data from the NAPL dissolution phase (0 - 32.8 PV, Section 5.3.2), but not from the pulse of VC-amended influent medium (see Section 5.3.4). Approximately 83% of the PCE initially present in the NAPL source zone was recovered in the column effluent (Figure 5.5A), primarily as *cis*-DCE (Table 5.1). This recovery is lower than expected since several lines of evidence indicate that the mixed-NAPL became completely depleted of PCE. A number of factors may have contributed to the mass balance discrepancy: (i) observations of side port septa swelling suggests PCE mass was lost via sorption to septa, especially in Port 1, (ii) visually observed mobilization of the mixed-NAPL around the bottom end-cap of the column removed some NAPL from the column, (iii) some NAPL was unintentionally, but unavoidably, removed from the column during side port sampling events, and (iv) partitioning into residual hexadecane and/or discrete gas bubbles formed within the column during operation may have acted as a sink for *cis*-DCE, VC, and ethene. Mass losses by these mechanisms could not be accurately quantified. These factors highlight the complexity of the column system and imply that the observed mass recovery is not unreasonable.

Table 5.1 Cumulative chlorinated ethene recoveries and mass transfer enhancement factors for the column experiment [a].

Parameter	**μmol**	**%**
Initial PCE Loading	16,319	–
Total Chlorinated Ethenes Recovered	13,486	82.6
PCE Recovered	295	1.8
TCE Recovered	87	0.5
cis-DCE Recovered	12,263	75.1
trans-DCE Recovered	29	0.2
VC Recovered	791	4.9
Ethene Recovered	21	0.1
Expected Abiotic PCE Recovered	2,610	16.0
Enhancement Factors		
Maximum Mass Transfer Enhancement Factor	21	
Cumulative Mass Transfer Enhancement Factor	5.2	

[a] Calculated with data from the NAPL dissolution phase (0-32.8 PV, Section 5.3.2), but not from the pulse of VC-amended influent medium (see Section 5.3.4)

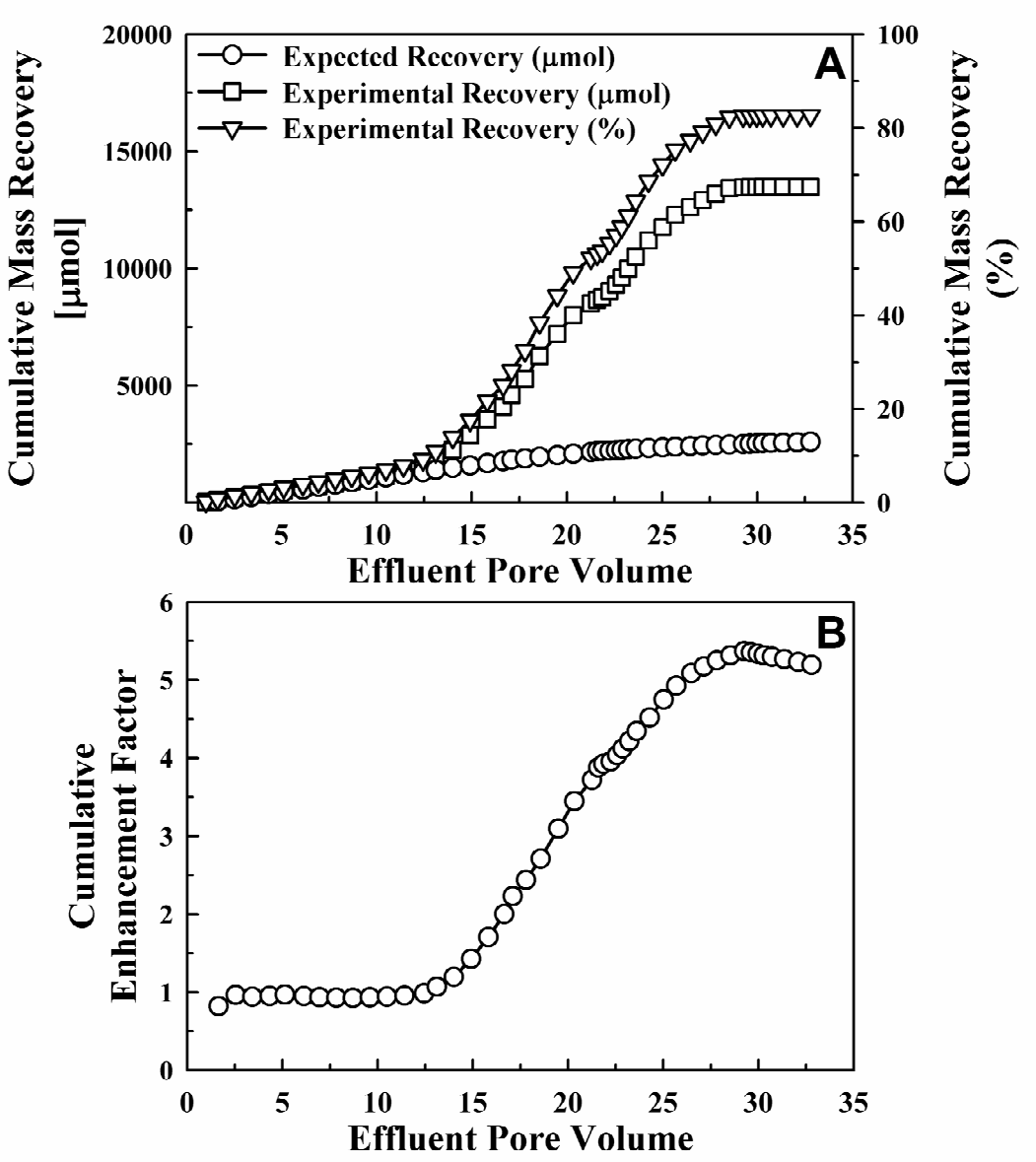

Figure 5.5 (A) Cumulative chlorinated ethene mass and percent recoveries and (B) cumulative mass transfer enhancement factor for the column experiment. See the text for details on how the expected (abiotic) recovery and the cumulative mass transfer enhancement factors were calculated.

Although some contaminant mass was unaccounted for, the observed (experimental) recovery of 83% is still significantly greater than the expected mass recovery under abiotic conditions (16%; see Table 5.1) (see Appendix A for more information on these calculations). The expected abiotic mass recovery was estimated by assuming an initial aqueous PCE solubility of ~300 μM, accounting for changing NAPL composition with PCE mass depletion (i.e., PCE mass depletion lowered the PCE mole fraction in the mixed-NAPL; see Appendix A), and assuming equilibrium mass transfer at both operational flow rates. Initially (<12 PV), the cumulative experimental and expected abiotic mass recoveries were almost identical (see Figure 5.5A). This is consistent with the data shown in Figure 5.2, which also indicates little bioenhanced dissolution at < 12 PV. At ~12 PV, the experimental mass recovery increased significantly faster than the expected abiotic recovery, indicating bioenhanced dissolution and suggesting microbial colonization of the source zone. To quantify the extent of the enhancement, a cumulative mass transfer enhancement factor was calculated by dividing the experimental mass recovery by the expected mass recovery (see Appendix A). As shown in Figure 5.5B, the cumulative mass transfer enhancement factor was approximately unity (i.e., no enhancement) for the first 12 PV of column operation. After this time, the cumulative mass transfer enhancement rapidly increased before leveling off and reaching a final value of 5.2 at 32.8 PV. An alternative way of calculating mass transfer enhancement is by comparing the experimental effluent concentration to the abiotic (expected) effluent concentration for a given effluent sample (see Appendix A). Enhancement factors calculated in this manner (termed maximum herein) are the ones most commonly reported in the literature and are always higher than cumulative

enhancement factors. For the current experiment, 21-fold maximum enhancement of mass transfer was observed utilizing this alternative definition. This maximum enhancement occurred at a single time point (24.2 PV; Figure 5.2). The enhancement factors are consistent with those observed during the *S. multivorans* mixed-NAPL column experiment and are comparable to the enhancement factors reported in the literature (see Chapter 2, Table 2.5) (*1*-*8*).

5.3.4 Vinyl Chloride (VC) Pulse

As discussed above, only limited amounts (<30 μM) of ethene were observed in during the NAPL dissolution phase of the column experiment. To assess the performance of VC-to-ethene dechlorinating organisms (i.e., *Dehalococcoides* strains) and to determine if conditions within the column were conducive to significant dechlorination of VC to ethene, the influent medium was amended with VC and effluent samples were taken to monitor VC elution and/or ethene production. The pulse of VC-amended medium was initiated at 32.8 PV, after the depletion of PCE from the mixed-NAPL and a decline in chlorinated ethene and ethene effluent concentrations to < 5 μM. The pulse lasted for ~1.1 pore volumes, resulting in the addition of approximately 34.6 μmol of VC to the column. The effluent concentrations of VC and ethene following the pulse of VC-amended medium are shown in the inset of Figure 5.2A.

As shown in Figure 5.2A, effluent VC and ethene concentrations began to increase over background levels at 34.5 PV of column operation (1.7 pore volumes after the initiation of the VC pulse). VC concentrations increased to a maximum value of 26.1 μM at 35.2 PV before returning to background levels by 35.9 PV. Ethene concentrations

(53.9 μM at 35.6 PV) were observed at approximately twice the level of VC, and ethene remained above background levels for much longer than VC. For both VC and ethene, the delay in VC and ethene breakthrough and the spreading (widening) of the breakthrough curves indicate that some VC and ethene retardation occurred in the column. The retardation was attributed to partitioning of VC and ethene into both residual NAPL (hexadecane) and discrete gas bubbles formed within the column during operation. Approximately 97.4% of the chlorinated ethene mass associated with the VC pulse was recovered in the effluent, with 75.6% of the total VC added to the column recovered as ethene in the effluent and the remaining 21.8% recovered as VC. Calculation of these recoveries corrected for the background levels of VC and ethene, which were assumed to be 1.3 μM and 4.2 μM, respectively, based on effluent concentrations prior to the VC pulse. The results of the VC pulse indicate that ethene formation within the column was possible and provide further evidence that the lack of significant ethene production throughout most of the column experiment was likely due to inhibition by polychlorinated ethenes (i.e., the presence of significant amounts of *cis*-DCE).

5.3.5 Final Microbial Distribution

At the conclusion of the experiment, the final microbial distribution was determined with both solid-phase (sand) samples (Figure 5.6A) and side port (liquid) samples (Figure 5.6B). The solid-phase samples were taken after termination of column operation (at 38.5 PV), while the side port samples were taken at 38.4 PV. Although high numbers of *Geobacter* and *Dehalococcoides* cells were detected in the source zone and over the entire length of the column in the solid-phase and aqueous phase samples

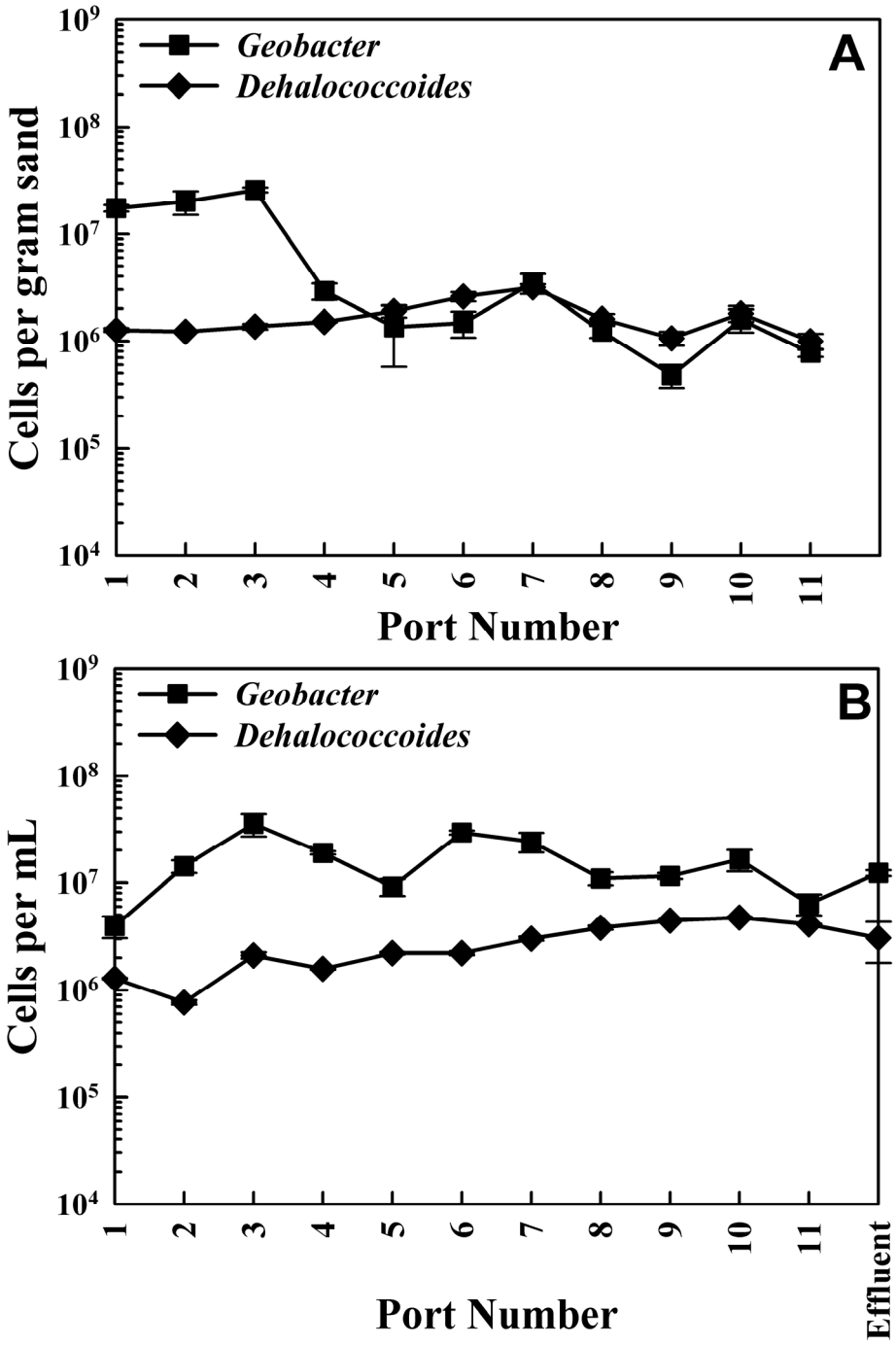

Figure 5.6 Microbial distribution of *Geobacter* and *Dehalococcoides* determined with (A) solid-phase (sand) samples and (B) side port (liquid) samples. The solid-phase samples were taken after column dissection at 38.5 PV, and the side port samples were taken after 38.4 PV of column operation. Port 1 is nearest the influent and in the source zone while Port 11 is nearest the effluent. Effluent concentrations taken at the same time are shown in (B) for comparison.

(Figures 5.6A and 5.6B), differences between the profiles were observed. For example, *Geobacter* organisms were detected at 1.8 x 10^7 to 2.6 x 10^7 cells per gram of sand in the source zone (Ports 1-3; Figure 5.6A). In the down gradient plume region (Ports 4-11), the number of *Geobacter* cells was approximately 1-2 orders-of-magnitude lower (4.8 x 10^5 to 3.5 x 10^6 cells per gram sand) than in the source zone (Figure 5.6A). Therefore, the solid-phase (sand) samples indicate that *Geobacter* organisms not only grew within the source zone but also attached to the porous medium within the source zone. This trend was not observed in the side port (liquid) samples (Figure 5.6B), where *Geobacter* cell numbers ranged from 3.9 x 10^6 to 3.5 x 10^7 per mL. In contrast to the profiles for *Geobacter*, the profiles for *Dehalococcoides* from the solid-phase (sand) and side port (liquid) samples were relatively similar to one another (Figures 5.6A and 5.6B). Additional studies are needed to evaluate the role of microbial attachment and possibly biofilm formation in bioenhanced dissolution and in promoting complete contaminant detoxification (i.e., ethene formation). The differences between the liquid and solid-phase profiles observed here also suggest that the form of environmental sample (aquifer solids or groundwater) might influence bioremediation monitoring and assessment with molecular biological tools (e.g., qPCR); additional work is needed to evaluate the differences between liquid and solid-phase samples for accurate microbial analysis.

5.4 Summary and Conclusions

Although previous studies have demonstrated biological activity within NAPL source zones, the contributions of individual dechlorinating populations to bioenhanced

dissolution are currently unknown. The involvement of key dechlorinating populations in enhanced contaminant dissolution, therefore, was explored with a 1-D continuous-flow column inoculated with BDI-SZ, a PCE-to-ethene dechlorinating microbial consortium containing multiple *Dehalococcoides* strains and two PCE-to-*cis*-DCE dechlorinating populations (*Geobacter lovleyi* strain SZ and a *Dehalobacter* species). Interestingly, the results suggest that the *Dehalobacter* population did not survive the initial stages of column operation (i.e., column packing) and, therefore, played a limited role, if any, in PCE dechlorination and bioenhanced dissolution. In contrast, growth of *Geobacter* and *Dehalococcoides* organisms was directly linked to the observed 5.2-fold cumulative bioenhanced dissolution, which is comparable to the dissolution enhancements observed with *S. multivorans* (Chapter 4) and in previous experiments (*1-8*).

Results from both chemical (e.g., chlorinated ethene) and qPCR analyses indicate that *Geobacter* and *Dehalococcoides* organisms were present and active in the mixed-NAPL (0.25/0.75 mol/mol PCE dissolved in hexadecane) source zone. These results corroborate the findings presented in Chapter 4 and suggest that successful source zone colonization by dechlorinating organisms is possible. *Geobacter* cell numbers were typically 1-3 orders-of-magnitude higher than *Dehalococcoides* cell numbers in samples from either the side ports or the column effluent. Spatial and temporal changes in both populations tracked closely with changes in the production of PCE dechlorination products (i.e., *cis*-DCE and VC). Interestingly, *Dehalococcoides* cell numbers generally increased 1-2 orders-of-magnitude over the length of the column, often correlating to increases in VC concentrations.

Despite the observed growth of both dechlorinating populations, PCE dechlorination generally stalled at *cis*-DCE, which reached concentrations as high as 2,600 μM. Similar dechlorination stalls have been reported in the literature (*1*-*8*). In this study, increasing the column residence time from 1.1 days to 2.8 days increased VC formation (up from < 50 μM to 300 μM), suggesting that the column residence time was insufficient to achieve significant dechlorination beyond *cis*-DCE. Ethene production was not observed until PCE was depleted from the mixed-NAPL and *cis*-DCE concentrations were reduced to low levels (< 10 μM), suggesting that *cis*-DCE may be inhibitory to complete dechlorination. Results from the pulse of VC-amended influent medium indicated that ethene formation within the column was possible and provide further evidence that the lack of ethene production throughout most of the column experiment was likely due to inhibition (i.e., the presence of significant amounts of *cis*-DCE). Accumulation of *cis*-DCE, therefore, might limit complete detoxification of PCE to ethene unless system residence times are sufficiently long for sequential conversion of *cis*-DCE to VC before VC conversion to ethene. Future studies should identify the mechanisms limiting complete PCE detoxification during source zone bioremediation and consider alternative remediation strategies, such as using biological activity as a tertiary (polishing) step following physical-chemical treatment (see Chapter 6).

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CHAPTER 6

EFFECTS OF THE NONIONIC SURFACTANT TWEEN 80 ON MICROBIAL REDUCTIVE DECHLORINATION OF CHLORINATED ETHENES

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6.1 Abstract

Recent field studies have indicated synergistic effects of coupling microbial reductive dechlorination with physical-chemical remediation (e.g., surfactant flushing) of dense non-aqueous phase liquid (DNAPL) source zones. This study explored chlorinated ethene (e.g., tetrachloroethene [PCE]) dechlorination in the presence of 50 - 5,000 mg/L Tween 80, a nonionic surfactant employed in source zone remediation. Tween 80 did not inhibit dechlorination by four pure PCE-to-*cis*-1,2-dichloroethene (*cis*-DCE) or PCE-to-trichloroethene (TCE) dechlorinating cultures. In contrast, *cis*-DCE-dechlorinating *Dehalococcoides* isolates (strain BAV1 and strain FL2) failed to dechlorinate in the presence of Tween 80. Bio-Dechlor INOCULUM (BDI), a PCE-to-ethene dechlorinating consortium, produced *cis*-DCE in the presence of Tween 80, further suggesting that Tween 80 inhibits dechlorination by *Dehalococcoides* organisms. Quantitative real-time PCR analysis applied to BDI revealed that the number of *Dehalococcoides* cells decayed exponentially ($R^2 = 0.85$) according to the Chick-Watson disinfection model (pseudo first-order decay rate of 0.13 ± 0.02 day^{-1}) from an initial value of $6.6 \pm 1.5 \times 10^8$ to $1.3 \pm$

0.8×10^5 per mL of culture after 58 days of exposure to 250 mg/L Tween 80. Although Tween 80 exposure prevented ethene formation and reduced *Dehalococcoides* cell numbers, *Dehalococcoides* organisms remained viable, and dechlorination activity past *cis*-DCE was recovered following the removal of Tween 80. These findings suggest that sequential Tween 80 flushing followed by microbial reductive dechlorination is a promising strategy for remediation of chlorinated ethene-impacted source zones.

6.2 Introduction

Chlorinated solvents, such as tetrachloroethene (PCE) and trichloroethene (TCE), are common groundwater contaminants that are frequently encountered as dense non-aqueous phase liquids (DNAPLs) in aquifer formations (*1*). Subsurface environments that contain DNAPL, either as residual ganglia or high saturation pools, represent long-term threats to environmental and public health since contaminant elution from these source zones can occur for decades (*2*). Several *in situ* remediation technologies have been developed and employed for treatment of DNAPL source zones, including physical--chemical processes such as surfactant flushing, *in situ* chemical oxidation, and thermal treatment, and biotic approaches including biostimulation and bioaugmentation (*1*). The objective of physical-chemical remedies is to reduce remediation times by aggressively removing or destroying contaminant mass within the source zone. Although significant amounts (60-90%) of the contaminant mass can be removed using physical-chemical technologies, short-term environmental risks may not be significantly reduced since post-treatment contaminant concentrations emanating from treated DNAPL source zones can

still exceed regulatory limits (*3*). Anaerobic bioremediation of chlorinated ethenes is mediated by bacteria that obtain energy for growth from reductive dechlorination reactions that detoxify the parent compound to benign ethene (i.e., [de]chlororespiration). Several bacterial isolates transform PCE to TCE (e.g., *Desulfitobacterium* sp. strain Viet1) or *cis*-1,2-dichloroethene (*cis*-DCE) (e.g., *Dehalobacter* spp., *Desulfuromonas* spp., *Sulfurospirillum* spp., *Desulfitobacterium* spp., and *Geobacter lovleyi* strain SZ (*4,5*)), while dechlorination past *cis*-DCE to vinyl chloride (VC) and ethene appears to be restricted to some members of the *Dehalococcoides* group (*6-8*). Bioremediation within PCE-DNAPL source zones faces many technical challenges, including (i) efficient supply of suitable electron donors to the dechlorinating bacteria (*9*), (ii) bioclogging in the vicinity of PCE DNAPL (*10,11*), (iii) toxicity of high PCE concentrations to dechlorinating bacteria (*12*), and (iv) incomplete dechlorination and formation of undesirable intermediates (e.g., *cis*-DCE, VC) as opposed to complete dechlorination of PCE to ethene (*11,13-17*).

Recent field studies indicated that coupling microbial reductive dechlorination with physical-chemical remediation may overcome the challenges associated with each technology when used in isolation (*18,19*). In a staged treatment scenario, the physical-chemical remedy removes significant contaminant mass, and, in case of surfactant or co-solvent flushing, delivers electron donors that may stimulate microbial reductive dechlorination activity (*18-20*). Hence, in this sequential approach, reductive dechlorination acts as a "polishing" step that detoxifies residual contaminants, thereby reducing contaminant mass flux and controlling long-term plume development. At the Bachman Road site in Oscoda, Michigan, a pilot-scale field demonstration of surfactant

enhanced aquifer remediation (SEAR) was performed using a 60,000 mg/L solution of Tween 80 (a nonionic, food-grade surfactant) to solubilize and remove PCE DNAPL from the source zone (*18,21,22*). SEAR is based on the ability of surfactants to substantially increase the aqueous phase solubility of hydrophobic organic contaminants when applied at concentrations above the critical micelle concentration (CMC; 13 mg/L for Tween 80 at 25ºC (*23*)) (reviewed in references (*24,25*)). In the Bachman aquifer, elevated concentrations (> 2 orders-of-magnitude greater than pretreatment values) of PCE dechlorination products (e.g., *cis*-DCE), as well as elevated levels of organic acids (e.g., acetate), were observed in monitoring wells 450 days following the cessation of surfactant flushing (*18*). Fermentation of residual Tween 80 (detected at 50 – 2,750 mg/L 450 days after SEAR) provided suitable electron donors(s) that stimulated native microbial dechlorination activity in the oligotrophic aquifer (*18*). Similar findings have been observed following cosolvent (i.e., ethanol) flushing at another PCE-DNAPL contaminated site (*19,20*).

For coupled remediation of PCE-DNAPL source zones to be considered feasible, the effects of physical-chemical intervention on the viability and activity of key dechlorinating organisms must be understood. In batch laboratory experiments, Ramsburg et al. (*18*) found that Tween 80 (1,000 – 5,000 mg/L) did not alter the dechlorination performance of *Desulfuromonas michiganensis* strain BB1, a PCE-to-*cis*-DCE dechlorinator indistinguishable from dechlorinating strain BRS1 present at the Bachman Road site (*26*). Another study reported that Tween 80 (1,000 – 10,000 mg/L) reduced the rate of ethene formation in a PCE-to-ethene dechlorinating consortium, though Tween 80 was the least inhibitory of several other surfactants tested (*27*). Yeh et

al. (*28*) observed that Tween 80 inhibition of hexachlorobenzene reductive dechlorination by three mixed cultures was dependent on surfactant concentration, with no inhibition observed at 10 mg/L, decreased dechlorination rates at 200 mg/L, and complete inhibition of dechlorination at 1,000 mg/L. These observations suggest that Tween 80 effects on dechlorinators are complex and insufficiently explored. Understanding the effects of surfactants on dechlorinating bacteria is vital for successful application of sequential remedies to a broad range of contaminated sites. Therefore, the objective of this research was to determine the influence of Tween 80 on key chlorinated ethene-dechlorinating bacteria at surfactant concentrations similar to those observed following SEAR at the Bachman Road site.

6.3 Materials and Methods

6.3.1 Chemicals

Tween 80 (polyoxyethylene [20] sorbitan monooleate) was obtained from Uniqema (New Castle, Deleware) and used without further purification. Tween 80 has an average molecular weight of 1,310 g/mole (see Figure 2.3) (*25*). PCE (≥99.9%) and TCE (≥99.5%) were purchased from Sigma-Aldrich Co. (St. Louis, Missouri). *cis*-DCE (99.9%) and *trans*-1,2-dichloroethene (*trans*–DCE, 99.9%) were obtained from Supelco Co. (Bellefonte, Pennsylvania). Gaseous VC (≥99.5%) was obtained from Fluka Chemical Corp. (Ronkonkoma, New York), and ethene (99.5%) was purchased from Scott Specialty Gases (Durham, North Carolina). All of the other chemicals used were reagent grade or better unless otherwise specified.

6.3.2 Cultures and Medium Preparation

The following pure cultures were used in this study: *Sulfurospirillum multivorans* (DSM 12446, (*29*)), *Geobacter lovleyi* sp. strain SZ (DSM 17278, (*5*)), culture "*Clostridium bifermentans* strain DPH-1" (*30*), *Desulfitobacterium* sp. strain Viet1 (*31,32*), *Dehalococcoides* sp. strain BAV1 (*7*), and *Dehalococcoides* sp. strain FL2 (*33*). The dechlorination activity of each isolate is listed in Table 6.1. In addition to the six pure cultures, two PCE-to-ethene dechlorinating mixed cultures were tested: the OW consortium (*34*) and Bio-Dechlor INOCULUM (BDI), a microbial consortium that has been successfully used for bioaugmentation at chlorinated ethene-contaminated sites (*35*).

Reduced anaerobic mineral salts medium for the pure cultures and the BDI consortium was prepared as described (*12,36,37*). Briefly, the medium contained the following (per liter): NaCl, 17.11 mmoles (1.0 g); $MgCl_2 \cdot 6H_2O$, 2.14 mmoles (0.5 g); NH_4Cl, 5.61 mmoles (0.3 g); KCl, 7.67 mmoles (0.3 g); $CaCl_2 \cdot 2H_2O$, 0.01 mmoles (0.015 g); KH_2PO_4, 0.2 mmoles (0.027 g); resazurin, 1 μmoles (0.25 mg); trace element solution A, 1 mL; trace element solution B, 1 mL; $Na_2S \cdot 9H_2O$, 0.05 mmoles (0.0120 g) (unless otherwise noted); anhydrous L-cysteine hydrochloride, 0.22 mmoles (0.035 g); dithiothreitol, 0.5 mmoles (0.0771 g); $NaHCO_3$, 30 mmoles (2.52 g); and TES (N-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid; $C_6H_{15}NO_6S$), 10 mmoles (2.292 g/L). For experiments with *Dehalococcoides* spp. strains BAV1 and FL2, the concentration of $Na_2S \cdot 9H_2O$ was increased to 0.2 mM, and the medium was amended with 0.5 mM Na_2SO_4. Trace element solutions A and B were prepared as described by Löffler et al. (*37*). After the medium had been boiled and cooled to room temperature under a stream of N_2, reductants (Na_2S, L-cysteine, and dithiothreitol) and $NaHCO_3$ were

Table 6.1 Summary of experimental parameters for each dechlorinating culture: dechlorination activity, electron donor, electron acceptor, headspace composition, and temperature.

Dechlorinating Culture[a]	Dechlorination Activity	Reference	Provided Electron Donor(s)[b]	Provided Electron Acceptor[c]	Headspace Composition (80%/20% [vol/vol])	Incubation Temperature
Sulfurospirillum multivorans	PCE to *cis*-DCE	(*29*)	Pyruvate (5 mM)	PCE (2 µL[d])	N_2/CO_2	22ºC
Geobacter lovleyi strain SZ	PCE to *cis*-DCE	(*5*)	Acetate (5 mM)	PCE (2 µL[d])	N_2/CO_2	22ºC
Culture "*Clostridium bifermentans* strain DPH-1"	PCE to *cis*-DCE	(*30*)	Citrate (5 mM) Yeast Extract (2 g/L) H_2 (5 mL)	PCE (4 µL[d])	N_2/CO_2	22ºC
Desulfitobacterium sp. strain Viet1	PCE to TCE	(*31,32*)	Pyruvate (5 mM)	PCE (2 µL[d])	N_2/CO_2	22ºC
Dehalococcoides sp. strain BAV1	*cis*-DCE to ethene	(*7*)	Headspace H_2 (48 mL)	*cis*-DCE (4 µL[e])	H_2/CO_2	30ºC
Dehalococcoides sp. strain FL2	TCE to VC	(*33*)	Headspace H_2 (48 mL)	TCE (4 µL[e])	H_2/CO_2	30ºC
Bio-Dechlor INOCULUM (BDI)	PCE to ethene	(*35*)	Lactate (5 mM)	PCE[f] (4 µL[d]) VC[f] (3 mL[g])	N_2/CO_2	30ºC[f], 22ºC[f]
OW Consortium	PCE to ethene	(*34*)	Headspace H_2 (16 mL)	PCE (1.25 µL[e])	H_2/CO_2	22ºC

[a] All cultures, except the OW Consortium, were grown in 160 mL serum bottles containing 100 mL ± 1 mL of mineral salts medium. The OW consortium was grown in 70 mL serum bottles containing ~50 mL of medium

[b] The aqueous phase concentration (mM) for each electron donor (except H_2) is provided in parentheses; for H_2, the volume (mL) amended to each serum bottle or provided in the headspace is given

[c] The volume of electron acceptor provided to each serum bottle is shown in parentheses

[d] A total of 2 or 4 µL of PCE were added as 0.1 or 0.2 mL of PCE dissolved in anoxic methanol (350 µL PCE per 15 mL of methanol; (*12*)), respectively

[e] The electron acceptor was provided in neat, liquid form

[f] For BDI, PCE was provided as the electron acceptor in the initial experiment (Table 6.2) and the incubation occurred at 30ºC. VC was provided as the electron acceptor in the subsequent experiments (Figure 6.3A; Figure 6.4) and the incubation occurred at 22ºC

[g] VC was provided as a gas

added and the pH of the medium was adjusted to 7.2 to 7.3 with CO_2 (*37*). Serum bottles (160 mL nominal volume, Wheaton Co. Millville, New Jersey) were flushed with a mixture of N_2/CO_2 or H_2/CO_2 (80%/20% [vol/vol]) (see Table 6.1) while medium (100 mL ± 1 mL) was dispensed. Bottles were sealed with Teflon-lined, gray butyl-rubber septa (#1014-4937, West Pharmaceuticals, Lionville, Pennsylvania) held in place with aluminum crimp caps (Wheaton). For experiments with *Dehalococcoides* spp. strains BAV1 and FL2, the bottles were sealed with black butyl-rubber stoppers (Geo-Microbial Technologies, Inc., Ochelata, Oklahoma). Vitamins were added to the medium after autoclaving from a sterile, anoxic stock solution (200-fold concentrated) to final concentrations as described (*38*), except that vitamin B_{12} was at twice the concentration. KH_2PO_4 was added to the medium after autoclaving from a sterile, anoxic stock solution (200 mM).

Reduced anaerobic mineral salts medium for the OW consortium was prepared as described (*34*). Briefly, the medium contained the following (per liter): $NaHCO_3$, 8 g; $Na_2S \cdot 9H_2O$, 0.3 g; $FeCl_2 \cdot 4H_2O$, 0.04 g; Trace Element Solution I, 10 mL; Trace Element Solution II, 10 mL; Basal Salts Solution, 10 mL. Trace Element Solution I contained 50 mg/L $ZnCl_2$, 50 mg/L $MnCl_2 \cdot 4H_2O$, 50 mg/L H_3BO_3, 250 mg/L $CoCl_2 \cdot 6H_2O$, 50 mg/L $NiCl_2 \cdot 6H_2O$, and 50 mg/L $Na_2MoO_4 \cdot 2H_2O$. Trace Element Solution II contained 1,000 mg/L $(NaPO_3)_{16}$, 250 mg/L KI, and 50 mg/L NH_4VO_3. The Basal Salts Solution contained 40 g/L KCl, 40 g/L $MgCl_2 \cdot 6H_2O$, 40 g/L NH_4Cl, 14 g/L KH_2PO_4, and 2.5 g/L $CaCl_2 \cdot 2H_2O$. The medium was prepared with reagent-grade chemicals and deionized water. Serum bottles (70 mL nominal volume, Wheaton) were flushed with ultra high purity (99.9%) N_2 for 12 min to remove oxygen before ~50 mL medium was

added under a stream of H_2/CO_2 (80%/20% [vol/vol]). The bottles were sealed with Teflon-lined butyl-rubber septa (Supelco, Bellefonte, Pennsylvania) held in place with aluminum crimp caps (Supelco).

6.3.2.1 Electron Donors and Carbon Sources. Electron donors and/or carbon sources were chosen based on strain specific requirements and added in excess (i.e., were not limiting dechlorination activity) from sterile, anoxic stock solutions by syringe using a 25 gauge needle to individual bottles (see Table 6.1): acetate (5 mM) for strain SZ; pyruvate (5 mM) for *S. multivorans* and strain Viet1; citrate (5 mM), yeast extract (2 g/L), and hydrogen (5 mL) for strain DPH-1; and lactate (5 mM) for BDI. Hydrogen, provided in the H_2/CO_2 (80%/20% [vol/vol]) headspace, served as electron donor for strains BAV1 and FL2 (see Table 6.1), and acetate (5 mM) served as the carbon source. Hydrogen, provided in the H_2/CO_2 (80%/20% [vol/vol]) headspace, served as electron donor for the OW consortium (see Table 6.1).

6.3.2.2 Electron Acceptors. Chlorinated ethenes were provided as terminal electron acceptors at non-inhibitory concentrations (see Chapter 3, reference (*12*)). The type of chlorinated ethene provided varied between cultures (see Table 6.1). Cultures of *S. multivorans,* strain SZ, and strain Viet1 received 0.1 mL of PCE dissolved in anoxic methanol (350 µL PCE per 15 mL of methanol; see Chapter 3, reference (*12*)), while cultures of strain DPH-1 received 0.2 mL of the PCE/methanol stock to achieve initial aqueous phase PCE concentrations of 150 and 300 µM, respectively. Cultures of strain BAV1 and strain FL2 received 4 µL of neat *cis*-DCE and TCE liquid, respectively. The

OW consortium received 1.25 µL of neat PCE. Neat solvents were added with 5 µL gastight syringes (model 95 with reproducibility [Chaney] adapters; Hamilton Co., Reno, Nevada). For BDI, PCE (0.2 mL of the PCE/methanol stock) or gaseous VC (3 mL) was utilized as the electron acceptor, as indicated.

6.3.3 Tween 80 Exposure

The pure cultures and BDI were incubated in the presence of Tween 80 at concentrations ranging from 50 to 5,000 mg/L (Table 6.2), which are similar to concentrations (50 – 2,750 mg/L) observed in the Bachman aquifer following surfactant flushing (*18*). Tween 80 was added from filter-sterilized anoxic stock solutions (23 or 50 g/L), which were prepared with anoxic, deionized water or reduced mineral salts medium in a glove box (Coy Laboratory Products, Ann Arbor, Michigan) filled with 95% N_2 and 5% H_2 [vol/vol]. Control cultures did not receive Tween 80 but instead were amended with equal volumes of sterile medium. Each vial was allowed to equilibrate for ≥2 days after the addition of a chlorinated ethene before inoculation with 2-5% (vol/vol) from actively dechlorinating stock cultures. Experiments were conducted in triplicate or replicate, independent assays incubated at 22ºC or 30ºC (see Table 6.1).

6.3.4 Biomass Effects on Tween 80 Inhibition

To explore the effect of biomass concentration on Tween 80 inhibition, the dechlorination performances of undiluted and 100-fold diluted OW cultures were compared. Aliquots (50 mL) from the 20-L stock culture of the OW consortium, which was grown in semi-batch mode with PCE as the electron acceptor and methanol as the

electron donor (*34*), were dispensed into triplicate, sterile, N_2-flushed serum bottles. The bottles were sparged with H_2/CO_2 (80%/20% [vol/vol]) to remove residual chlorinated ethenes. Aliquots (0.5 mL) were then diluted 100-fold into triplicate bottles containing reduced medium. Each bottle received PCE (1.25 µL) and Tween 80 (final concentration of 1,000 mg/L) to bring the total volume per vessel to 50 mL. Triplicate control cultures did not receive Tween 80, but instead received equal volumes of medium. All OW cultures were incubated at 22ºC. Particulate organic carbon (POC) measurements estimated steady-state biomass concentrations from stock cultures of both the OW and BDI consortia as described (*39*). To collect POC, samples were filtered through a glass microfiber filter (Whatman Inc., Florham Park, New Jersey) with 0.7 µm (nominal diameter) pores.

6.3.5 Effect of Tween 80 on *Dehalococcoides* Cell Numbers

To evaluate the effect of Tween 80 on *Dehalococcoides* cell numbers, BDI cultures were monitored under three different incubation conditions, each maintained at 22ºC. The BDI consortium was chosen for this experiment since it is a robust culture that is easily maintained in the laboratory, contains multiple *Dehalococcoides* strains (*40*), and has been successfully used in bioaugmentation field applications (*35*). Triplicate vessels were amended with 250 mg/L Tween 80. An additional set of triplicates did not receive VC or Tween 80 and served as electron acceptor-starved controls. Duplicate bottles without Tween 80 served as positive control cultures. Each culture vessel received 3 mL of sterile VC gas (unless otherwise indicated) as electron acceptor. Following a 2-day equilibration period, the vessels were inoculated with 15% (vol/vol)

from an actively dechlorinating BDI consortium grown with PCE as the electron acceptor. Before inoculation, 200 mL of BDI were placed into sterile, N_2-flushed serum bottles, and a filter-sterilized stream of N_2/CO_2 (80%/20% [vol/vol]) was bubbled through the cell suspension for 15 minutes to remove residual chlorinated ethenes. Before distribution of the inoculum to individual experimental bottles, a sample of the inoculum was collected for quantitative real-time PCR (qPCR) analysis. The experiment was repeated with a longer incubation time for the Tween 80-exposed treatment and the positive control bottles. Samples were taken periodically for DNA extraction and qPCR analysis (see Sections 6.3.7 and 6.3.8 for procedural details). The terms "16S rRNA gene copies" and "cell numbers" are per mL of culture fluid and are used interchangeably because the known *Dehalococcoides* organisms contain one 16S rRNA gene copy per genome (*41,42*).

6.3.6 Reversibility Experiments

To determine the reversibility of Tween 80 inhibition of *Dehalococcoides* organisms, aqueous samples (1 mL) were collected from triplicate bottles of the BDI consortium exposed to 250 mg/L Tween 80 for 21 days. Samples were also collected after 21 days of incubation from BDI cultures without surfactant (duplicate positive control cultures) or in the absence of chlorinated ethenes (triplicate electron acceptor-starved control cultures). Samples collected from duplicate BDI cultures exposed to filter-sterilized air for 21 days served as negative controls. The biomass was collected by centrifugation for 30 minutes at 4,300 × g using a microcentrifuge placed inside a glove box. The supernatant was decanted, and the biomass from 15 1-mL samples of culture

suspension was collected in the same tube. The resulting pellets were suspended (i.e., washed) in 1.5 mL of reduced mineral salts medium, and the centrifugation step was repeated. Each pellet was then suspended in 1 mL of medium and served as inoculum for Tween 80-free medium that had been equilibrated with 3 mL of VC. The cultures were then incubated at 22ºC. The pH of all vials was determined on Day 48 and adjusted with 6N NaOH to pH 7.2 ± 0.1, if necessary.

6.3.7 DNA Extraction

BDI biomass was collected periodically from 10 mL of culture fluid by centrifugation at 4°C for 30 minutes at 3,220 × g. All but ~1 mL of the supernatant was decanted, in which the bacterial cell pellet was suspended. Centrifugation was repeated at 16,000 × g at room temperature for 10 minutes. The supernatant was removed and the pellet was stored at -20°C until genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, California) following the modifications described by Ritalahti et al. (*40*). DNA was obtained in a final volume of 200 µL buffer AE (provided with the QIAamp DNA Mini Kit) and stored at -20°C until qPCR analysis.

Experiments were performed to determine the effects of Tween 80 on DNA extraction efficiency. BDI culture suspensions (6 mL each) were mixed with Tween 80 (60 µL of a 50 g/L stock solution to reach a final concentration of ~500 mg/L) and incubated for 10 minutes at room temperature. Control samples received 60 µL of reduced medium that did not contain Tween 80. DNA was extracted from three 2-mL samples from both the control and Tween 80 amended samples, and *Dehalococcoides* 16S rRNA genes were quantified by qPCR analysis.

6.3.8 Quantitative Real-Time PCR (qPCR) Analysis

qPCR analysis to quantify the *Dehalococcoides* 16S rRNA gene copies was performed using either TaqMan- or SYBR Green-based detection chemistries. The TaqMan-based assay was performed as described (*40*), using the primers Dhc1200F and Dhc1271R and TaqMan probe Dhc1240Probe that specifically target the *Dehalococcoides* 16S rRNA gene (*40,43*). Each optical PCR tube (Applied Biosystems, Foster City, California) contained 1x TaqMan universal PCR master mix (ABI), 300 nM probe, 300 nM of each primer, and 3 µL of template DNA (10 ng/µL - 40 ng/µL of community DNA for unknown samples [quantified by spectrophotometry at a wavelength of 260 nm (*40*)]) in a total reaction volume of 30 µL. PCR cycle parameters for the TaqMan-based approach were as follows: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 58°C. qPCR analysis utilizing the TaqMan probe was carried out in a ABI Prism 7700 Sequence Detection System. The SYBR Green-based assay used the same *Dehalococcoides*-specific primers as above. The reaction mix was amended with 2x QuantiTect SYBR Green PCR master mix (Qiagen), and the PCR cycle parameters were as follows: 2 min at 50°C, 15 min at 95°C followed by 40 cycles of 15 sec at 94°C, 1 minute at 60°C, and 30 sec at 72°C. qPCR analysis utilizing the SYBR Green-based approach was carried out in a ABI Prism 7000 Sequence Detection System. Sterile water was used for negative controls in both the TaqMan- and SYBR Green-based approaches to identify false positives. Standard curves were generated as described (*40*), and quantification (i.e., dynamic range, sensitivity, amplification efficiency) was similar between both the TaqMan- and SYBR Green-based approaches (data not shown). The terms "16S rRNA gene copies" and "cell numbers" are per mL of

culture fluid and are used interchangeably because the known *Dehalococcoides* organisms contain one 16S rRNA gene copy per genome (*41,42*).

6.3.9 Analytical Methods

Aqueous phase (1 mL) or headspace (0.1 mL) samples were collected periodically for chlorinated ethene and ethene quantification by gas chromatography. Aqueous phase samples were analyzed with a Hewlett-Packard (HP) 7694 headspace autosampler connected to a HP 6890 gas chromatograph (GC) equipped with a HP-624 column (60 m by 0.32 mm; film thickness, 1.8 μm nominal) and a flame ionization detector (FID) as described (see Chapter 3, reference (*12*)). Linear correlations between increasing Tween 80 concentrations and decreasing signal (i.e., peak area) for PCE and *cis*-DCE were used to correct measured PCE and *cis*-DCE concentrations during analytical detection with the HP 7694 headspace autosampler (*18*). The correction was <5% for both PCE and *cis*-DCE at the Tween 80 concentrations used in this study. Headspace samples were manually injected into the HP 6890 GC and analyzed as described (*26*). Standard calibration curves for chlorinated ethenes and ethene analysis were prepared as described (*12,44,45*). Analyte distribution between the aqueous phase and headspace were determined using Henry's law constants specific for each chlorinated ethene (*44*) or ethene (*46*). The presence of Tween 80 alters the equilibrium partitioning of PCE (*18*); therefore, the Henry's constant for PCE at various Tween 80 concentrations was adjusted as described in Kibbey et al. (*47*), assuming a PCE aqueous solubility of 200 mg/L (*48*) and a molar solubilization ratio (MSR) of 5.41 mol PCE/mol Tween 80 (*49*).

6.4 Results

6.4.1 Dechlorination Performance in the Presence of Tween 80

The dechlorination performance of both pure and mixed cultures in the presence of Tween 80 is summarized in Table 6.2. At the concentrations tested, Tween 80 had no effect on PCE-to-TCE and PCE-to-*cis*-DCE reductive dechlorination in all cultures tested (Table 6.2). Similar dechlorination performances and product patterns were observed in cultures with and without the surfactant. For example, Figure 6.1 shows PCE dechlorination to *cis*-DCE in cultures of culture "*Clostridium bifermentans* strain DPH-1" in the presence and absence of 500 mg/L Tween 80. In contrast, Tween 80 had a profound effect on *cis*-DCE dechlorination in cultures of *Dehalococcoides* sp. strain BAV1. As shown in Figure 6.2, the addition of 50 mg/L Tween 80 completely inhibited *cis*-DCE dechlorination, whereas control cultures without surfactant produced stoichiometric amounts of ethene. Tween 80-amended cultures did not show any *cis*-DCE dechlorination activity even after 2 months of incubation (data not shown). Similarly, *Dehalococcoides* sp. strain FL2 did not dechlorinate TCE in the presence of Tween 80, while control cultures dechlorinated TCE to mainly VC over the 34-day incubation period (Table 6.2). Replicate, independent experiments yielded similar results regardless of the Tween 80 concentrations tested (50-5,000 mg/L; see Table 6.2). The BDI consortium dechlorinated PCE to ethene in the absence of the surfactant, but *cis*-DCE was the dechlorination end product in the presence of Tween 80 (500-5,000 mg/L; Table 6.2). VC or ethene formation did not occur even after extended (147 days) incubation of BDI with Tween 80 (data not shown).

Table 6.2 Summary of dechlorination performance of pure and mixed cultures in the presence of Tween 80.

	Provided Electron Acceptor	Tween 80 Concentrations (mg/L)	Dechlorination End Product	
			Control[a]	With Tween 80
Pure Cultures				
Desulfuromonas michiganensis strain BB1[b]	PCE	1000, 5000	*cis*-DCE	*cis*-DCE
Sulfurospirillum multivorans	PCE	500, 1000, 5000	*cis*-DCE	*cis*-DCE
Geobacter lovleyi strain SZ	PCE	500, 1000, 5000	*cis*-DCE	*cis*-DCE
Culture "*Clostridium bifermentans* strain DPH-1"	PCE	500	*cis*-DCE	*cis*-DCE
Desulfitobacterium sp. strain Viet1	PCE	500, 1000, 5000	TCE	TCE
Dehalococcoides sp. strain BAV1	*cis*-DCE	50, 250	ethene	—[c]
Dehalococcoides sp. strain FL2	TCE	250	VC	—[c]
Mixed Cultures				
Bio-Dechlor INOCULUM (BDI)	PCE	500, 1000, 5000	ethene	*cis*-DCE
OW Consortium	PCE	1000	ethene	*cis*-DCE/ethene[d]

[a] Cultures incubated without Tween 80
[b] Data presented in Ramsburg et al. (*18*)
[c] No dechlorination observed
[d] The biomass concentration affected product formation (see text for details)

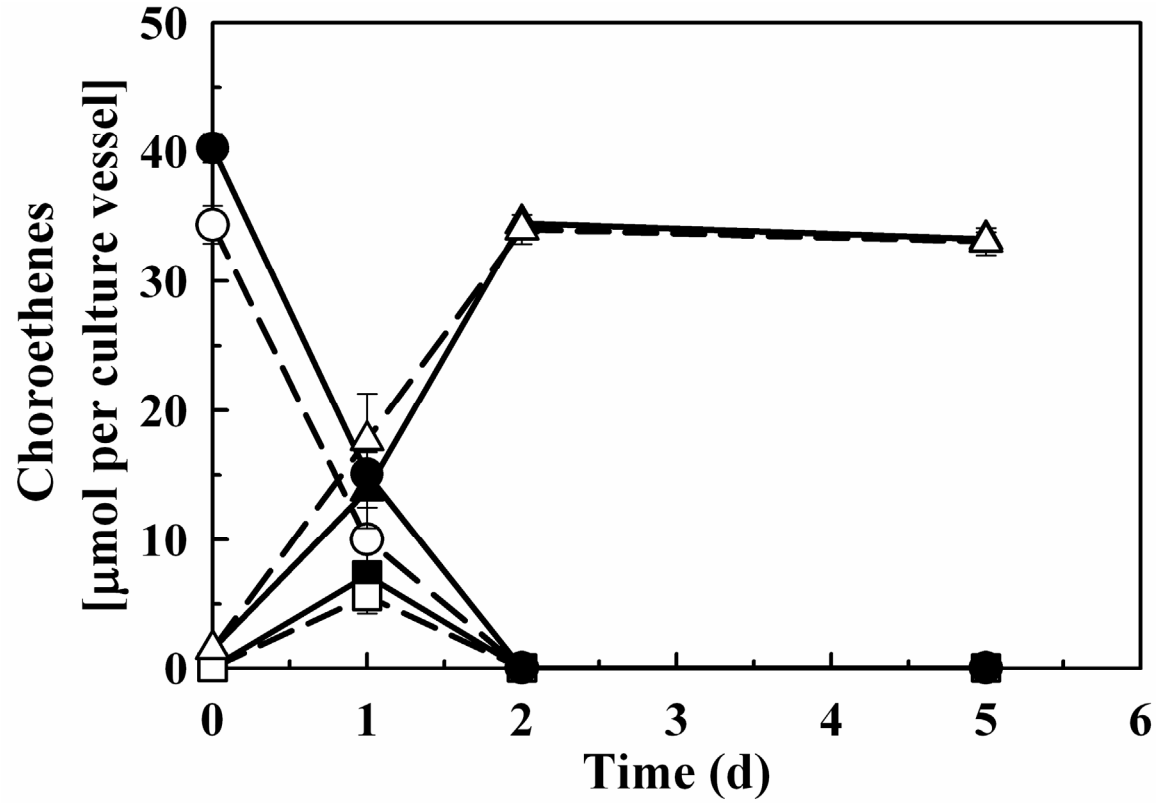

Figure 6.1 The effect of Tween 80 on the reductive dechlorination activity of culture "*Clostridium bifermentans* strain DPH-1". Experimental cultures contained 500 mg/L Tween 80 (open symbols, dashed lines) while the control cultures did not contain Tween 80 (closed symbols, solid lines). Symbols: ●, PCE; ■, TCE; ▲, *cis*-DCE. All data points represent average values from triplicate cultures, and error bars represent one standard deviation. When error bars are not visible, they are smaller than and therefore hidden behind the data symbols.

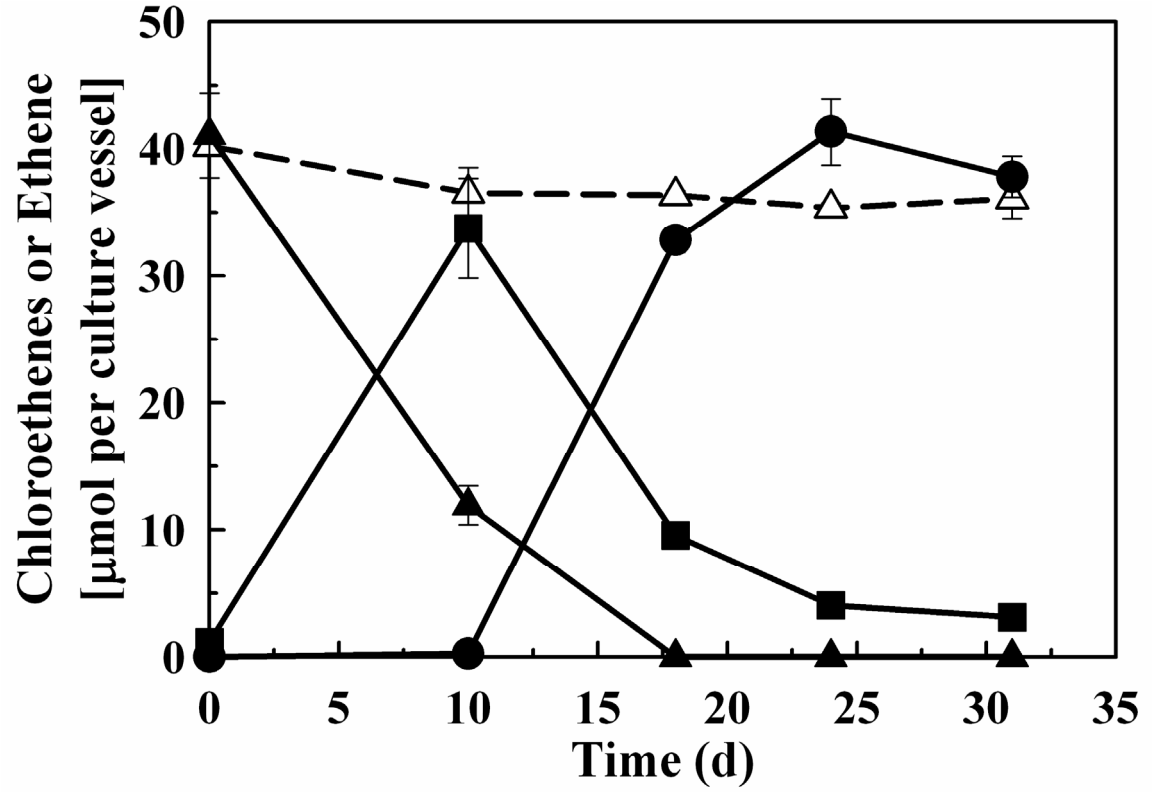

Figure 6.2 The effect of Tween 80 on the reductive dechlorination activity of *Dehalococcoides* sp. strain BAV1. Experimental cultures contained 50 mg/L Tween 80 (open symbols, dashed line) while the control cultures did not contain Tween 80 (closed symbols, solid lines). Symbols: ▲, *cis*-DCE; ■, VC; ●, Ethene. All data points represent average values from triplicate cultures, and error bars represent one standard deviation. When error bars are not visible, they are smaller than and therefore hidden behind the data symbols.

6.4.2 Biomass Effects on Tween 80 Inhibition of Reductive Dechlorination

The biomass concentration maintained in the OW consortium was 20-fold higher compared with the BDI consortium (i.e., 820 ± 50 mg POC/L vs 40 ± 5 mg POC/L). Phase contrast light microscopy confirmed that the OW consortium contained more microbial cells than BDI and revealed large cell aggregates ranging in size from ~10 μm to greater than 300 μm (data not shown). The aggregates consisted of bacteria with diverse morphologies dominated by small cocci and filamentous bacteria similar in morphology to *Methanosaeta* (data not shown) (*50*). The BDI consortium contained few cell aggregates, which were ≤ 10 μm in diameter, dominated by small cocci, and did not contain filamentous bacteria. In contrast to the observations made with BDI described above, Tween 80 (1,000 mg/L) did not inhibit dechlorination beyond *cis*-DCE with undiluted OW consortium (Table 6.2); both the surfactant-free control and Tween 80-amended undiluted OW cultures dechlorinated PCE to ethene following 2 days of incubation (data not shown). In experiments that used 100-fold dilutions of the OW consortium, TCE (2 mol% of the total chlorinated ethenes) and *cis*-DCE (98 mol%) accumulated in the presence of Tween 80 after 8 days of incubation, and no further dechlorination to VC and ethene occurred even after extended incubation periods (42 days). The 100-fold dilution affected the dechlorination performance of the OW consortium in the absence of Tween 80 (i.e., the surfactant-free control cultures); VC (87 mol% of total chlorinated ethenes and ethene) was the main product following a 42-day incubation period. Although dilution of the OW consortium affected complete dechlorination of PCE to ethene in the surfactant-free control cultures, comparison of dechlorination performances in the 100-fold diluted cultures (i.e., surfactant-free vs.

Tween 80-amended cultures) suggested that Tween 80 inhibited *cis*-DCE dechlorination to VC and ethene.

6.4.3 Effect of Tween 80 on *Dehalococcoides* Cell Numbers

The Tween 80-exposed BDI cultures initially dechlorinated VC to small amounts of ethene (8 ± 1 μmol), but additional dechlorination of VC and production of ethene was not observed after Day 4 (Figure 6.3A). In the absence of Tween 80, VC was dechlorinated by Day 21 with concomitant production of ethene (Figure 6.3A). In an independent experiment, the Tween 80-exposed BDI cultures failed to dechlorinate VC, and ethene was not detected even after 58 days of incubation (data not shown). The *Dehalococcoides* cell numbers steadily declined from $1.7 \pm 1.1 \times 10^8$ to $2.0 \pm 0.8 \times 10^6$ per mL during the 21 days of Tween 80 exposure (Figures 6.3B and 6.3C) and from $6.6 \pm 1.5 \times 10^8$ to $1.3 \pm 0.8 \times 10^5$ per mL following the 58-day Tween 80 exposure (Figure 6.3C). For the control cultures incubated without Tween 80, the number of *Dehalococcoides* cells increased from $1.7 \pm 1.1 \times 10^8$ per mL on Day 0 to a maximum of $1.0 \pm 0.6 \times 10^9$ per mL on Day 17 during dechlorination of VC to ethene (Figure 6.3B). The cell numbers for the electron acceptor-starved control cultures ranged from $1.4 \pm 0.4 \times 10^8$ to $3.3 \pm 1.0 \times 10^8$ cells per mL during the 21-day incubation period (Figure 6.3B). Regression analysis ($R^2 = 0.85$) of both the 21-day and 58-day incubations revealed that the *Dehalococcoides* cell numbers per mL decreased exponentially (pseudo first-order decay rate of 0.13 ± 0.02 day^{-1}) with time during Tween 80 exposure (Figure 6.3C). At the concentrations tested, Tween 80 had no effect on DNA extraction or PCR efficacy

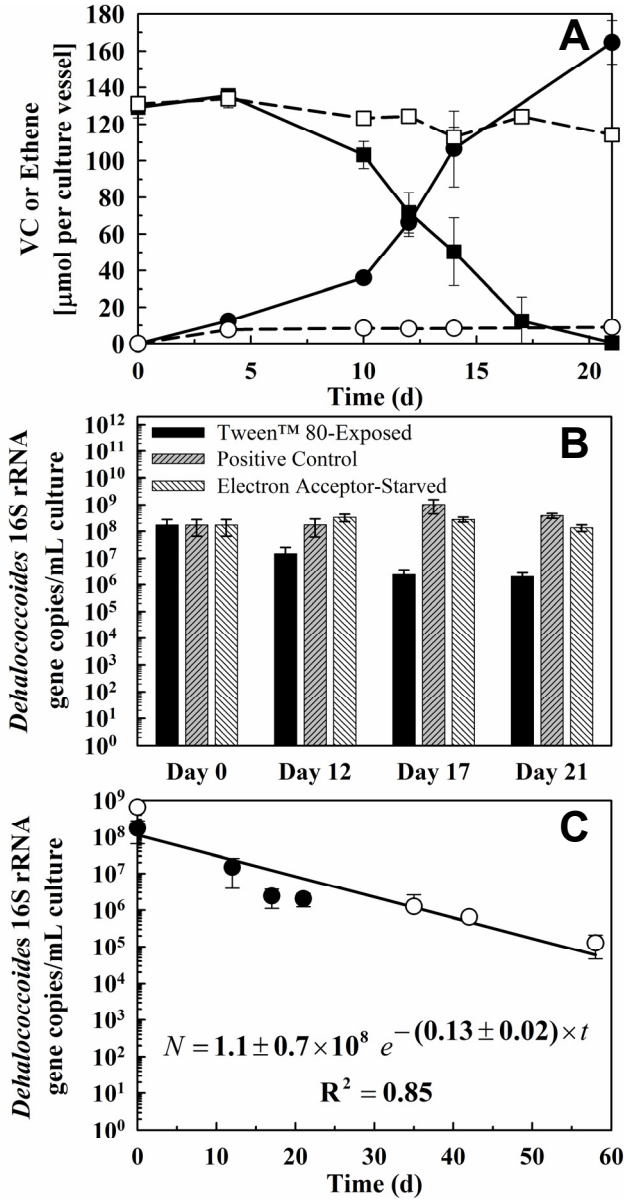

Figure 6.3 The effect of Tween 80 on *Dehalococcoides* cell numbers. (A) VC dechlorination by the BDI consortium in the presence (250 mg/L; open symbols, dashed lines) or absence (closed symbols, solid lines) of Tween 80. Symbols: ■, VC; ●, Ethene. (B) qPCR analysis of *Dehalococcoides* 16S rRNA gene copy numbers during incubation under three treatments. (C) qPCR analysis showing an exponential decrease in the number of *Dehalococcoides* organisms during exposure to 250 mg/L Tween 80. The solid line in (C) represents regression of the data from both the 21-day exposure (closed symbols) and the 58-day exposure (open symbols); the line appears linear on the logarithmic axis. *N* represents the number of *Dehalococcoides* cells per mL at time *t*. Error bars represent one standard deviation. When error bars are not visible, they are smaller than and therefore hidden behind the data symbols.

(data not shown), indicating that the decreased cell numbers measured with qPCR analysis were not due to experimental biases introduced by the surfactant.

6.4.4 Reversibility Experiments

The Tween 80-exposed, washed BDI cell suspensions dechlorinated VC to ethene in Tween 80-free medium. Ethene production began on Day 24 and neared completion by Day 30 (Figure 6.4). A much shorter lag time of 3 days was observed in the control cultures that had not experienced Tween 80 exposure (Figure 6.4). Ethene production also occurred in suspensions of washed, electron-acceptor starved cells (representative results are shown in Figure 6.4). Although the dechlorination performance of the triplicate cultures of the washed, electron-acceptor starved cells varied, complete dechlorination of VC to ethene occurred by Day 52 in all replicates (data not shown). The negative control cultures (i.e., washed, oxygen-exposed cells) did not dechlorinate VC or produce ethene (data not shown).

6.5 Discussion

The presence of Tween 80 did not affect the dechlorination performance of both gram-positive and gram-negative PCE-to-TCE or PCE-to-*cis*-DCE dechlorinating isolates. The tested isolates included all major genera of PCE-to-*cis*-DCE dechlorinators except *Dehalobacter* spp. However, *Dehalobacter* spp. were detected in consortia OW (*34*) and BDI (unpublished data), and the ability of both consortia to dechlorinate PCE to *cis*-DCE in the presence of surfactant suggests that *Dehalobacter* spp. are not affected by

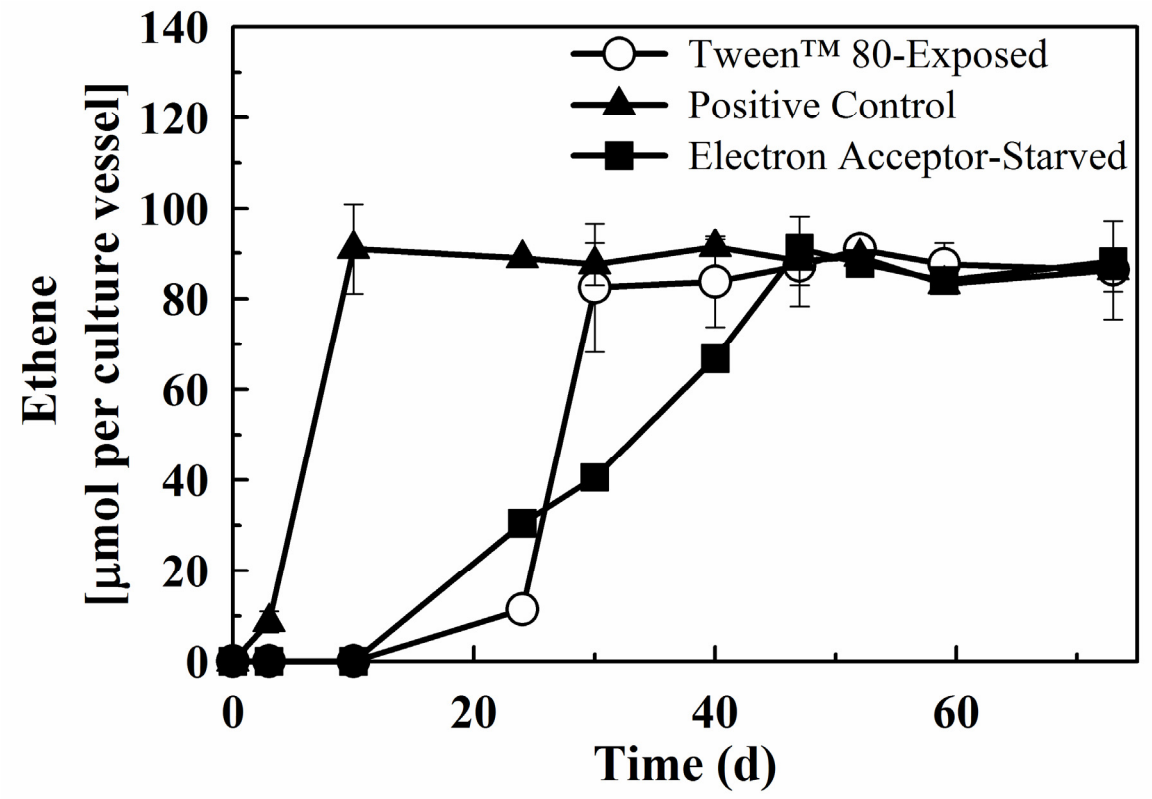

Figure 6.4 Reversibility experiment showing ethene production by washed cell suspensions of Bio-Dechlor INOCULUM (BDI) in identical sets of fresh Tween 80-free medium. Representative data sets show values from one replicate (electron acceptor-starved cultures) or average values from duplicate (positive control) or triplicate (Tween 80-exposed) cultures. Error bars represent one standard deviation.

Tween 80. Apparently, Tween 80 does not inhibit PCE-to-TCE and PCE-to-*cis*-DCE dechlorinators, at least at surfactant concentrations similar to those observed at the Bachman Road site following SEAR (i.e., < 5,000 mg/L Tween 80) (*18*).

In contrast, Tween 80 severely inhibited dechlorination by *Dehalococcoides* organisms in both pure and mixed cultures and caused a decline in cell numbers. The sequestration of chlorinated ethenes within surfactant micelles (*51*) may have limited the bioavailability of the terminal electron acceptor required to sustain the energy metabolism in *Dehalococcoides* cells. However, the cell numbers remained constant in electron acceptor-starved control cultures (which were not exposed to Tween 80), suggesting a direct effect of the surfactant on the *Dehalococcoides* cells. Obviously, *Dehalococcoides* organisms respond differently to Tween 80 as compared to the gram-positive and gram-negative PCE-to-TCE and PCE-to-*cis*-DCE dechlorinators, which were not affected by Tween 80 at the concentrations tested. Although the mode of Tween 80 inhibition is unclear, it is conceivable that the surfactant affects *Dehalococcoides* membrane functions and ultimately causes cell death and lysis. Phylogenetically, *Dehalococcoides* are deeply branching green non-sulfur bacteria (*Chloroflexi*) with an unusual cell wall structure and membrane composition (*6*), which may be responsible for the observed Tween 80 effects. For instance, furan phospholipid fatty acids, which are not typically found in bacteria, were detected in *Dehalococcoides* (*52*), and an archaeal-type gene (*ino1*), which has limited distribution in bacteria and may be involved in the synthesis of cellular envelope components, was found in the genome of *Dehalococcoides ethenogenes* (*53,54*).

The decrease in *Dehalococcoides* cell numbers during Tween 80 exposure were fit ($R^2 = 0.85$) to the Chick-Watson disinfection model ($N = N_o e^{-kt}$), where N and N_o are the *Dehalococcoides* 16S rRNA gene copies per mL culture at time t and initially, respectively, and k is the pseudo first-order reaction rate (*55,56*). The predictable nature of the decline in *Dehalococcoides* cells during exposure to Tween 80 allows the incorporation of decay rates in mathematical models (e.g., UTCHEM (*57*)) used to evaluate and design remedial treatment options (e.g., SEAR followed by microbial reductive dechlorination). Since the pseudo first-order decay rate, k, is likely a function of surfactant type and concentration, type of microorganism, and environmental parameters (e.g., pH, temperature), values determined herein only apply to the experimental conditions used in this study. Future research should evaluate the effect of environmental parameters, including surfactant concentrations, degradation, sorption, and dilution on k to improve incorporation of this parameter into remedial design models.

Despite the evidence that Tween 80 inhibits dechlorination mediated by *Dehalococcoides* organisms and reduces their survival, a study by McGuire and Hughes (*27*) suggested that *Dehalococcoides* can dechlorinate in the presence of Tween 80. In their study, a dechlorinating consortium transformed PCE to VC and ethene when amended with 1,000 – 10,000 mg/L Tween 80 (*27*). The results presented herein with the OW consortium, which was derived from the same source as the dechlorinating consortium used by McGuire and Hughes (*27,34*), also indicated ethene formation in the presence of 1,000 mg/L Tween 80. Microscopic analysis and POC measurements showed that the OW consortium contained at least 20-fold more biomass than the BDI consortium, which did not dechlorinate PCE beyond *cis*-DCE in the presence of Tween

80. Interestingly, the OW consortium failed to dechlorinate beyond *cis*-DCE in the 100-fold diluted cultures. These results suggest that the amount of biomass and/or type of microbes present in the OW consortium reduced the inhibitory effect of Tween 80 on *Dehalococcoides* organisms. One possible mechanism to explain this observation is sorption of Tween 80 to microbial cells. In high biomass systems (e.g., the undiluted OW consortium with 820 ± 50 mg POC/L), the mass of surfactant associated with microbial cells may be below inhibitory levels. In lower biomass systems (e.g., BDI with 40 ± 5 mg POC/L and the 100-fold diluted OW consortium with 8.2 ± 0.5 mg POC/L), the mass of surfactant associated with microbial cells may exceed a tolerable threshold and cause inhibition. For example, Yeh and Pavlostathis (*58*) determined that sorption of a similar surfactant (Tween 60) to biomass from a hexachlorobenzene-dechlorinating consortium reached a maximum value of 1.182 g surfactant/g biomass organic carbon. It is likely that the sorption properties of Tween 80 and Tween 60 are similar because the surfactants are structurally related and follow Langmuir-type sorption isotherms (*21,58*). Based on the Langmuir parameters reported by Yeh and Pavlostathis (*58*) and the experimental conditions (e.g., initial [applied] surfactant concentration), surfactant sorption to biomass was estimated at ~0.8 and ~1.0 g surfactant/g biomass POC for the undiluted and 100-fold diluted OW consortium, respectively. In both systems, the bulk aqueous-phase surfactant concentration did not decrease below the surfactant's CMC; therefore, the lack of inhibition in high biomass systems is likely related to lower amounts of surfactant associated with the biomass as opposed to a reduction of bulk aqueous-phase surfactant concentrations. At contaminated sites, the amount of surfactant available for sorption to biomass is controlled not only by the amount of biomass present

but also by the physical-chemical properties of the soil (e.g., organic matter content). Sorption of Tween 80 to aquifer material will not only reduce the amount of surfactant available to associate with microbial cells (e.g., *Dehalococcoides* organisms) but also provide a long-term source of reducing equivalents (i.e., electron donors) to sustain reductive dechlorination (*18*). Surfactant sorption properties affect remedial performance in multiple ways and should be carefully considered in combined treatment applications. Another possible mechanism to explain the increased tolerance of consortium OW to the surfactant is the occurrence of large cell aggregates that may shield and protect *Dehalococcoides* organisms from exposure to and interaction with Tween 80. Association of *Dehalococcoides* organisms with other microbes may provide similar protection from exposure to Tween 80 in aquifer systems. Both proposed mechanisms, surfactant sorption and protection received within cell aggregates, may operate independently or in tandem. Future research is needed to explore the complex interactions between cell titers (i.e., biomass), aggregate and/or biofilm formation, surfactant sorption, and the ability of *Dehalococcoides* organisms to tolerate surfactant exposure under *in situ* conditions. These studies should include Tween 80 and other surfactants applicable for SEAR to develop a comprehensive understanding of the effects of these compounds on key dechlorinators (e.g., *Dehalococcoides*).

Even though Tween 80 negatively affected *Dehalococcoides* organisms in pure and enriched cultures, dechlorination activity past *cis*-DCE was recovered following the removal of the surfactant, which simulates conditions following SEAR and subsequent surfactant degradation, dilution, sorption, and transport. The effect(s) of Tween 80 on *Dehalococcoides* organisms was reversible because at least a fraction of the cells

remained viable during exposure to the surfactant, suggesting that the *Dehalococcoides* population can rebound following SEAR. These findings, combined with (i) the tolerance of PCE-to-TCE and PCE-to-*cis*-DCE dechlorinators to Tween 80, (ii) the reduced influence of Tween 80 on *Dehalococcoides* cells in systems with high biomass and/or complex microbial assemblages, and (iii) the degradation of Tween 80 to organic acids and alcohols that serve as sources of reducing equivalents supporting reductive processes (*18*), suggest that staged treatment incorporating Tween 80 flushing followed by microbial reductive dechlorination is a promising remedial strategy for chlorinated ethene-impacted source zones. The feasibility of these "treatment train" approaches for source zone remediation needs to be validated in controlled field studies that monitor the fate and transport of Tween 80 (and other surfactants applicable for SEAR), surfactant fermentation and the production of organic acids, alcohols and hydrogen, and the activity, distribution, and abundance of key microorganisms during and after source zone flushing.

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CHAPTER 7

DETECTION AND QUANTIFICATION OF *GEOBACTER LOVLEYI* STRAIN SZ: IMPLICATIONS FOR BIOREMEDIATION AT TETRACHLOROETHENE- (PCE-) AND URANIUM-IMPACTED SITES

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7.1 Abstract

Geobacter lovleyi strain SZ reduces U(VI) to U(IV) and is the first member of the metal-reducing *Geobacter* group capable of using tetrachloroethene (PCE) as a growth-supporting electron acceptor. Direct and nested PCR with specific 16S rRNA gene-targeted primer pairs distinguished strain SZ from other known chlorinated ethene-dechlorinating bacteria and closely related *Geobacter* isolates, including its closest cultured relative, *G. thiogenes.* Detection limits for direct and nested PCR were approximately 1 x 10^6 and 1 x 10^4 16S rRNA gene copies per μL of template DNA, respectively. A quantitative real-time PCR (qPCR) approach increased the sensitivity to as few as 30 16S rRNA gene copies per μL of template DNA but was less specific. Melting curve analysis and comparison of the shapes of amplification plots identified false positive signals and distinguished strain SZ from *G. thiogenes* in qPCR analysis. Application of the specific tools detected strain SZ-like amplicons in PCE-dechlorinating

consortia, including the bioaugmentation consortium KB-1, and two chlorinated ethene-impacted groundwater samples. Strain SZ-like amplicons were also detected in 13 of 22 groundwater samples following biostimulation at the uranium- and chlorinated solvent-contaminated Integrated Field-Scale Subsurface Research Challenge (IFC) site in Oak Ridge, Tennessee. The numbers of strain SZ-like cells increased from below detection to $2.3 \pm 0.1 \times 10^7$ per liter groundwater, suggesting that strain SZ-like organisms contribute to contaminant transformation. The *G. lovleyi* strain SZ-specific tools may prove useful for monitoring bioremediation efforts at uranium- and/or chlorinated solvent-impacted sites such as the Oak Ridge IFC site.

7.2 Introduction

Chlorinated ethenes such as tetrachloroethene (PCE) and trichloroethene (TCE) are common groundwater pollutants due to their extensive use in industrial and military applications (*1*). Uranium, typically present in the environment as soluble U(VI), has been released into the environment as a consequence of weapons grade uranium production and milling (*2,3*). Uranium processing typically required chlorinated solvents, such as PCE and TCE; therefore uranium and chlorinated ethene contamination often coexist (e.g., at the Integrated Field-Scale Subsurface Research Challenge [IFC] site [formerly the Field Research Center] in Oak Ridge, Tennessee [http://www.esd.ornl.gov/nabirfrc]) (*4,5*). Due to negative human health impacts, both chlorinated ethenes and uranium are regulated by the U.S. Environmental Protection Agency (EPA). Considerable effort has been devoted to elucidate the fate of these

contaminants and to identify microbial populations involved in their transformation and detoxification (*6-8*). Although a number of phylogenetically diverse PCE- and U(VI)-reducing bacteria have been isolated from various environments (*6,7,9,10*), only the recently described isolate *Geobacter lovleyi* strain SZ shares the ability to reductively dechlorinate PCE and reduce soluble U(VI) to sparingly soluble U(IV) (*11*). Interestingly, strain SZ reduces PCE and U(VI) concomitantly (*11*); therefore, strain SZ is a promising candidate for bioremediation at mixed waste sites such as the Oak Ridge IFC site. Based on the analysis of nearly complete 16S rRNA gene sequences, strain SZ and *G. thiogenes* (a trichloroacetate-to-dichloroacetate dechlorinator) form a putative dechlorinating clade within the *Geobacteraceae* (*11*). The goals of this study were to design specific 16S rRNA gene-based approaches to distinguish the PCE-dechlorinating and U(VI)-reducing *G. lovleyi* strain SZ from other *Geobacter* species, including its closest cultured relative, *G. thiogenes*, and to demonstrate the value of quantitative analyses to assess the environmental distribution and abundance of strain SZ-like *Geobacter* spp. in uranium- and chlorinated ethene-contaminated sites.

7.3 Materials and Methods

7.3.1 Sources of DNA

DNA was isolated from pure cultures, PCE-dechlorinating enrichment cultures, bioaugmentation consortia, and groundwater and aquifer materials from contaminated sites. The pure cultures included *G. lovleyi* strain SZ (DSM 17278, (*11*)), *G. thiogenes* (formerly *Trichlorobacter thiogenes*) (ATCC BAA-34, (*12,13*)), *G. sulfurreducens* (DSM

12127, (*14*)), *G. metallireducens* (DSM 7210, (*15*)), *Desulfuromonas michiganensis* strain BB1 (DSM 15941, (*16*)), *Anaeromyxobacter dehalogenans* strain 2CP-C (ATCC BAA-259, (*17*)), *Sulfurospirillum* (formerly *Dehalospirillum) multivorans* (DSM 12446, (*18,19*)), *Dehalococcoides* sp. strain FL2 (*20*), *Dehalococcoides* sp. strain BAV1 (*21*), and *Desulfitobacterium* sp. strain Viet1 (*22,23*). The *Geobacter* isolates were grown as described by Sung et al. (*11*). Strain BB1 and strain 2CP-C were grown as described by Sung et al. (*16*) and Sanford et al. (*17*), respectively, and all other pure cultures were grown as described by Amos et al. (*24*). Rebecca C. Daprato kindly provided genomic DNA and a plasmid containing a single copy of the 16S rRNA gene of *Dehalobacter restrictus* (DSM 9455, (*25*)). Genomic DNA was obtained from three PCE-to-ethene dechlorinating enrichment cultures derived from chlorinated ethene-contaminated aquifer materials (the Hydrite site, Cottage Grove, Wisconsin; the TRW site, Minerva, Ohio; and the FMC site, San Jose, California) (*26*); one PCE-to-*cis*-1,2-dichloroethene (*cis*-DCE) dechlorinating enrichment culture derived from TCE-contaminated aquifer materials collected at East Gate Disposal Yard in Ft. Lewis, Washington (*27*); and one PCE-to-ethene dechlorinating mixed culture derived from uncontaminated river sediment (Kalamazoo River sediment, Michigan) (*26*). Bio-Dechlor INOCULUM (BDI), a PCE-to-ethene dechlorinating bioaugmentation inoculum, was grown as described (*24*) to obtain genomic DNA. Genomic DNA of the commercially available bioaugmentation inoculum KB-1 (*28,29*) was kindly provided by Melanie Duhamel, University of Toronto. DNA was also recovered from chlorinated ethene-contaminated aquifer material: Ft. Lewis aquifer solids and groundwater collected from two monitoring wells within a TCE plume from an industrial site near Milledgeville, Georgia.

Twenty-two groundwater samples were obtained from the uranium-contaminated Oak Ridge IFC site. Samples were collected from wells FW016 and FW029 of Area 1 (*30*) and well TPB16 of Area 2. Area 3 groundwater samples were from the unconditioned and non-stimulated control well FW106 (May 27, 2004, (*31*)) and from a pilot-scale uranium bioreduction demonstration plot (*5,31*). The pilot-scale demonstration used a nested-cell approach comprising a biostimulated inner recirculation loop that received electron donor (i.e., ethanol) and an outer recirculation loop that minimized interactions between the low pH and high nitrate aquifer groundwater and the chemically and biologically conditioned groundwater in the biostimulated, inner loop (*5,31*). Substantial reduction of U(VI) to U(IV) occurred in the inner recirculation loop following aquifer conditioning and ethanol biostimulation (*5,31*). The four-well recirculation system consisted of the outer loop injection well (FW024), the outer loop extraction well (FW103), the inner loop injection well (FW104), and inner loop extraction well (FW026). The system also contained three multilevel sampling (MLS) wells: FW100, FW101, and FW102. Specific MLS well depths (level 1, 15.24 m below ground surface [bgs]; level 2, 13.7 m bgs; level 3, 12.19 m bgs; and level 4, 10.67 m bgs) are designated as outlined in Wu et al. (*5*). For example, FW101-4 represents well FW101, level 4. See Wu et al. (*5*) for a complete description of the recirculation loops and MLS wells. Periodic addition of ethanol to the inner recirculation loop began on January 7, 2004 (*31*). Groundwater samples were obtained on four occasions during the first six months of periodic biostimulation: February 2, 2004 (wells FW103, FW100-2, FW100-3, FW101-3, and FW104); February 5, 2004 (well FW024); May 27, 2004 (wells FW026 and FW101-2); and June 16, 2004 (well FW102-3). Additional samples were

also obtained 1-1.5 years after continued biostimulation: May 25, 2005 (well FW104), August 4, 2005 (wells FW104 and FW102-2); and August 5, 2005 (wells FW026, FW101-1, FW102-1, FW102-4, FW100-1, and FW100-4). Eleven of the twenty-two samples came from wells (FW029, FW104, FW026, FW101-3, FW101-1, FW101-2, FW102-2, and FW102-3) located in subsurface regions affected by biostimulation (i.e., ethanol addition), while the remaining samples came from wells (FW016, TPB16, FW106, FW024, FW103, FW100-2, FW100-3, FW102-1, FW102-4, FW100-1, and FW100-4) located in regions not influenced or only marginally influenced by biostimulation (*5,30,31*).

7.3.2 DNA Isolation

Genomic DNA was extracted from 10 mL of chlorinated ethene-dechlorinating pure and mixed cultures, as well as from cultures of *G. thiogenes*, *G. metallireducens*, and *G. sulfurreducens*, using the QIAamp DNA Mini Kit (Qiagen, Valencia, California) with modifications as described (*32*). For qPCR method development and optimization, biomass was collected and genomic DNA extracted from 10 mL (*G. lovleyi* strain SZ, *G. thiogenes*, and *G. sulfurreducens*) or 5 mL (*G. metallireducens*) of culture fluid as described (see *Supporting Information* in reference (*24*)). For qPCR standards, the 16S rRNA gene of strain SZ was cloned into plasmid Topo-TA pCR2.1 (Invitrogen, Carlsbad, CA) (*33*); plasmids containing the 16S rRNA gene of strain SZ (pSZ16S) were extracted from *E. coli* host cells using the QIAprep Spin Miniprep Kit (Qiagen) (*32*). Community genomic DNA was isolated as described (*34*) from 1.8 to 1,715 liters of Oak Ridge IFC site groundwater and collected in final volumes of 10-450 µL; for qPCR, the DNA was

diluted 1:10 or 1:20 before analysis. Genomic DNA was isolated using the MoBio Water kit (0.2 μm pore size; Jefferson City, Missouri) from 1 liter of groundwater from the TCE-contaminated site in Milledgeville, Georgia. Genomic DNA was extracted from 10 g of Ft. Lewis aquifer solids using the PowerMax Soil DNA Isolation Kit (MoBio) and from 40 mL of a TCE-fed enrichment culture established with Ft. Lewis aquifer material using the MoBio Ultra Clean Water DNA Kit, according to the manufacturers' protocols. Purified DNA was stored at -20°C until analysis.

7.3.3 Primer Design and PCR Conditions

Specific PCR primer pairs were designed using the Oligo Design and Analysis Tools (www.idtdna.com) based on the nearly complete 16S rRNA gene sequence of strain SZ (accession number AY914177). The 16S rRNA gene sequences of strain SZ and other *Desulfuromonadales* including *G. thiogenes* (AF223382), *G. sulfurreducens* (U13928), *G. metallireducens* (L07834), and *Desulfuromonas michiganensis* strain BB1 (AF357915) were aligned using the MegAlign program of the Lasergene software package (DNA Star Inc., Madison, Wisconsin). The sequences of the selected primers, Geo196F and Geo999R, and mismatches between the primer sequences and the sequences of 16S rRNA gene fragments from closely related organisms are shown in Table 7.1. The expected size of Geo196F/Geo999R amplicon is 820 bp. This amplicon size is generally unsuitable for quantitative real-time PCR (qPCR) (*35*); therefore, another primer pair generating a shorter amplicon was needed for qPCR analysis. Thus, the *Geobacteraceae*-targeted primer Geo564F described by Cummings et al. (*36*) was modified (i.e., reverse complement, W [which equals A/T] in the fourth position was

Table 7.1 Primers used to detect and quantify *Geobacter lovleyi* strain SZ, and alignment of 16S rRNA gene sequence fragments of strain SZ with corresponding regions of closely related species.

Organism	Primer Sequence Alignment (5' → 3') [a]		
	Geo196F [b]	Geo535R [b]	Geo999R [b]
G. lovleyi	GAATATGCTCCTGATTC	TAAATCCGAACAACGCTT	ACCCTCTACTTTCATAG
G. thiogenes	GAATATGCTCTTGATCT	TAAATCCGAACAACGCTT	ACCCCTCGCTTTCACGA
G. metallireducens	TTCGGGCCTTTTGTCAC	TAATTCCGAACAACGCTT	ACCCCCTACTTTCATAG
G. sulfurreducens	TTCGGGCCTCCTGTCTT	TAATTCCGAACAACGCTT	ACCCCTCCGTTTCGGGA
D. michiganensis	TCGGGTCCTACTGTCAT	TAATTCCGAACAACGCTT	ACCCCTATGTTTCCATA

[a] Gray highlights indicate mismatches.
[b] The primers correspond to the following positions on the *E. coli* 16S rRNA gene sequence (U00096.2): Geo196F, 208-224 bp; Geo535R, 563-546 bp; and Geo999R, 1025-1009 bp. The positions were determined using the MegAlign program of the Lasergene software package (DNA Star Inc., Madison, Wisconsin).

changed to A, the primer was shortened at the 5' end by 1 bp) to produce primer Geo535R (Table 7.1). The expected size of Geo196F/Geo535R amplicons is 357 bp.

BLAST analysis suggested primer specificity to the target sequences. To verify primer specificity, purified genomic DNA of close relatives (*G. thiogenes*, *G. sulfurreducens*, and *G. metallireducens*) and selected reductively dechlorinating species (*D. michiganensis* strain BB1, *Anaeromyxobacter dehalogenans* strain 2CP-C, *Sulfurospirillum multivorans*, *Dehalobacter restrictus*, *Dehalococcoides* sp. strain FL2, *Dehalococcoides* sp. strain BAV1, and *Desulfitobacterium* sp. strain Viet1) was subjected to PCR with both Geo196F/Geo999R and Geo196F/Geo535R primer pairs. The reaction mixture contained 2 µL of 10x PCR reaction buffer (Applied Biosystems, Foster City, California), 2 mM $MgCl_2$, 0.13 mg of bovine serum albumin/mL (Promega, Madison, Wisconsin), 200 µM of each dNTP (Applied Biosystems), 2.5 U of ampliTaq polymerase (Gibco BRL, Gaithersburg, Maryland), 100 nM of each primer, and 1 µL of template DNA (10-20 ng/µL) in a total reaction volume of 20 µL. The annealing temperature for the PCR reaction varied from 45 to 57ºC (Eppendorf Mastercycler Gradient 5331, Eppendorf, Hamburg, Germany); the optimum annealing temperatures were 50 and 53ºC for primer pairs Geo196F/Geo535R and Geo196F/Geo999R, respectively. The following temperature program was used for the designed primer pairs: 94ºC for 2.2 min, 30 cycles of 94ºC for 30 sec, 50ºC or 53ºC for 45 sec, and 72ºC for 2.2 min, followed by 6 min at 72ºC. For nested PCR, an initial amplification was performed with universal bacterial primer pair 8F and 1514R followed by a second round of PCR with the strain SZ-specific primer pairs using 1:50 dilutions of the amplicons obtained in the first round of PCR as template DNA (*37*). PCR products were visualized on a 1%

agarose gel in Tris-acetate-EDTA buffer stained in an aqueous ethidium bromide solution (1 μg/mL). Select amplicons obtained with the Geo196F/Geo999R primer pair were purified (QIAquick PCR purification kit; Qiagen) and partially sequenced using an ABI 3100 genetic analyzer (Applied Biosystems).

The detection limits (i.e., sensitivities) of direct and nested PCR with the Geo196F/Geo999R primer pair were determined by performing PCR on a 10-fold dilution series of *G. lovleyi* strain SZ genomic DNA. The gene targets in the highest dilution were quantified via qPCR analysis, and the DNA concentrations in subsequent dilutions were calculated based on this estimate. The reported detection limits are the lowest dilutions that yielded a visible band on ethidium bromide stained gels.

7.3.4 Quantitative Real-Time PCR (qPCR) Analysis

qPCR analysis to quantify *G. lovleyi* strain SZ was performed using SYBR Green-based detection chemistry and the Geo196F/Geo535R primer pair. The reaction mixture contained 15 μL of Power SYBR Green PCR master mix (Applied Biosystems), 300 nM of each primer, and 3 μL of template DNA in a total reaction volume of 30 μL. The PCR temperature program was as follows: 2 min at 50ºC, 15 min at 95ºC followed by 40 cycles of 30 sec at 94ºC, 30 sec at 50ºC, and 30 sec at 72ºC. qPCR was carried out in a Applied Biosystems 7500 Fast Real-Time PCR System, and melting curve analysis was performed with the default settings of the ABI software from 67ºC to 95ºC following the completion of each qPCR. Standard curves were generated following the procedure outlined in Ritalahti et al. (*32*) and used a 10-fold dilution series of quantified plasmid (concentration determined spectrophotometrically at 260 nm) carrying a single copy of

strain SZ's 16S rRNA gene (pSZ16S). The total length of the plasmid (3,931 bp) with the cloned 16S rRNA gene insert (1,534 bp) was 5,465 bp. To identify false positives, template DNA was replaced with sterile water (i.e., no template controls, NTCs). The genome of strain SZ contains two 16S rRNA gene copies (www.jgi.doe.gov); therefore, dividing gene copy numbers by a factor of two yielded cell numbers.

The specificity of the Geo196F/Geo535R primer pair in the qPCR approach was evaluated with triplicate samples of purified genomic DNA of *G. lovleyi* strain SZ (12.5 ng/μL), *G. thiogenes* (16.1 ng/μL), *G. sulfurreducens* (1.9 ng/μL), and *G. metallireducens* (6.9 ng/μL). DNA concentrations, shown in parentheses, were determined spectrophotometrically at 260 nm. The specificity of the primer pair was also tested with 1:10 and 1:100 dilutions of *G. thiogenes* genomic DNA.

To determine the influence of DNA from closely related species on the quantification of strain SZ, qPCR analysis was performed on DNA mixtures (see Table 7.2) with increasing amounts of *G. thiogenes* DNA. To determine if sample dilution alone affected strain SZ quantification, qPCR was performed on undiluted and 1:10 diluted strain SZ DNA.

7.4 Results and Discussion

7.4.1 Specific, Sensitive, and Quantitative Detection of Strain SZ

With strain SZ DNA as template, the designed primer pairs Geo196F/Geo999R and Geo196F/Geo535R yielded amplicons of the expected sizes (820 bp and 357 bp, respectively). Sequence analysis of amplicons obtained with the Geo196F/Geo999R

Table 7.2 Summary of the effect of *Geobacter thiogenes* DNA on qPCR estimation of *Geobacter lovleyi* cell numbers.

G. thiogenes DNA (ng/μL) [a]	Dilution of *G. lovleyi* strain SZ DNA [b]	*G. lovleyi* cells per mL ± SD [c]	N/N_o [d]
0	Undiluted (12.5 ng/μL)	$1.42 \pm 0.06 \times 10^8$	0.94
0	1:10	$1.59 \pm 0.15 \times 10^8$	1.06
0.32	1:10	$1.61 \pm 0.09 \times 10^8$	1.07
0.64	1:10	$1.73 \pm 0.05 \times 10^8$	1.15
0.97	1:10	$1.72 \pm 0.05 \times 10^8$	1.15
1.29	1:10	$1.73 \pm 0.10 \times 10^8$	1.15
1.61	1:10	$1.72 \pm 0.10 \times 10^8$	1.14
4.03	1:1.33	$2.10 \pm 0.19 \times 10^8$	1.39
5.36	1:1.5	$2.24 \pm 0.12 \times 10^8$	1.49
8.05	1:2	$2.68 \pm 0.19 \times 10^8$	1.78

[a] Calculated concentration after dilution of 16.1 ng/μL of *G. thiogenes* genomic DNA (estimated spectrophotometrically at 260 nm) with known volume(s) of strain SZ genomic DNA and/or sterile water.

[b] The dilution of 12.5 ng/μL (estimated spectrophotometrically at 260 nm) of genomic DNA of *G. lovleyi* strain SZ with known volume(s) of *G. thiogenes* genomic DNA and/or sterile water.

[c] The estimated number of *G. lovleyi* cells per mL of culture fluid (average of triplicate samples ± standard deviation [SD]) based on qPCR analysis. The estimate takes into account the dilution of strain SZ genomic DNA, the volume (10 mL) of culture from which DNA was extracted, the final volume (200 μL) of extracted DNA, and that two 16S rRNA gene copies are present on the genome of strain SZ.

[d] N/N_o represents the ratio of the estimated number (N) of *G. lovleyi* cells per mL culture for each sample (column 3) to the average (N_o; $1.50 \pm 0.11 \times 10^8$ cells per mL) of the two estimates in the absence of *G. thiogenes* DNA.

primer pair corroborated specific amplification. In direct and nested PCR, both primer pairs distinguished strain SZ from related *Desulfuromonadales* species (i.e., *G. thiogenes*, *G. sulfurreducens*, *G. metallireducens*, and *D. michiganensis* strain BB1 [see Figure 7.1A]) and other reductively dechlorinating bacteria (i.e., *Anaeromyxobacter dehalogenans* strain 2CP-C, *Sulfurospirillum multivorans*, *Dehalobacter restrictus*, *Dehalococcoides* sp. strain FL2, *Dehalococcoides* sp. strain BAV1, and *Desulfitobacterium* sp. strain Viet1). As shown in Figure 7.1B, direct PCR with the Geo196F/Geo999R primer pair required $>10^6$ 16S rRNA gene copies per µL of template DNA to produce a visible band in ethidium bromide stained agarose gels. The nested PCR approach was two orders of magnitude more sensitive (Figure 7.1B). Although the primer pair Geo196F/Geo999R did not achieve the sensitivity reported for other 16S rRNA gene-targeted primers (e.g., see ref (*37*)), the strain SZ-specific approach proved valuable in evaluating the distribution of strain SZ-like organisms in environmental samples (see below). With a quantification limit of ~30 16S rRNA gene copies per µL of template DNA, the qPCR approach was significantly more sensitive and produced linear standard curves ranging from $8.4 \times 10^1 - 8.4 \times 10^8$ 16S rRNA gene copies per PCR reaction ($R^2 = 0.9991$; amplification efficiency = 1.80 (*38*)). The qPCR quantification limit reported herein is consistent with the quantification limits reported for other qPCR target genes (e.g., *Dehalococcoides* 16S rRNA and reductive dehalogenase genes (*32*)) and slightly lower than the quantification limit observed with another *Geobacter*-targeted qPCR protocol (*29*). The latter method used primer pair Geo73f/Geo485r to target a *Geobacter* organism whose 16S rRNA gene sequence (AY780563) shares 99% identity to *G. lovleyi* strain SZ (*29*). This primer pair does not contain the discriminating

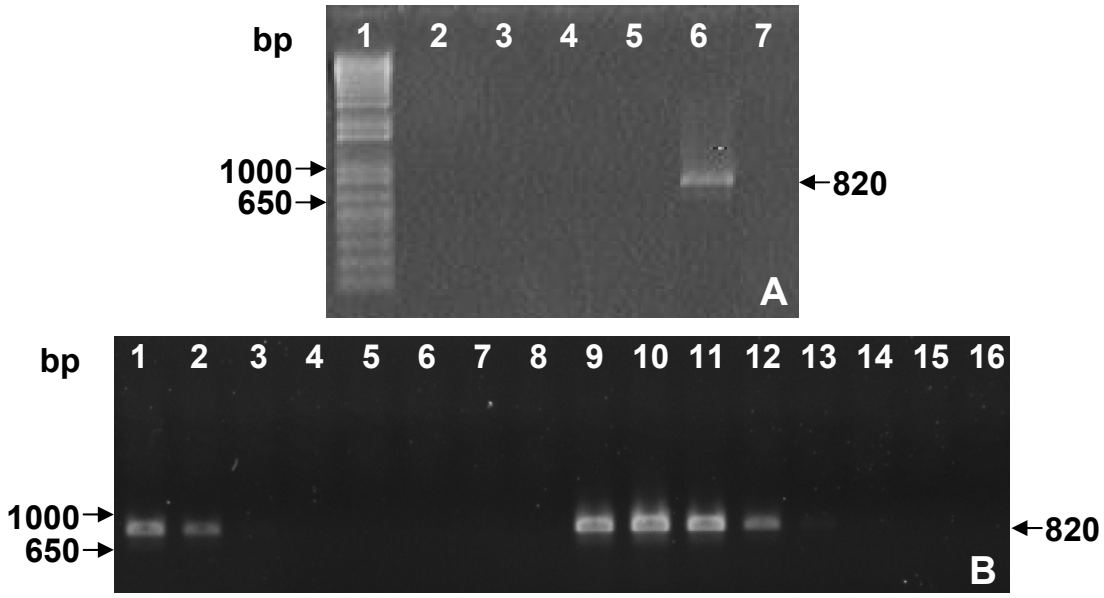

Figure 7.1 Specificity (A) and sensitivity (B) of primer pair Geo196F and Geo999R targeting the 16S rRNA gene of *Geobacter lovleyi* strain SZ. Purified genomic DNA was used as template for each PCR reaction. In (A): lane 1, DNA size marker (Invitrogen); lane 2, *G. thiogenes*; lane 3, *G. sulfurreducens;* lane 4, *G. metallireducens*; lane 5, *D. michiganensis* strain BB1; lane 6, *G. lovleyi* strain SZ; lane 7, water (NTC). In (B): direct (lanes 1-8) and nested (lanes 9-16) PCR with a ten-fold dilution series of genomic DNA of *G. lovleyi* strain SZ ranging from $1.33 \pm 0.22 \times 10^7$ (lanes 1 and 9) to $1.33 \pm 0.22 \times 10^0$ (lanes 8 and 16) 16S rRNA gene copies per μL DNA, as determined with qPCR analysis (see text for details). Portions of the gel picture in (B) were assembled electronically.

mismatches presented in Table 7.1, and hence, would not distinguish between *G. lovleyi* and *G. thiogenes*.

In qPCR analysis, the shapes of the amplification (i.e., fluorescent signal intensity) plots (not shown) and the melting curves (Figure 7.2) were similar for triplicate samples of genomic DNA from strain SZ as compared to the plasmid (pSZ16S) standards. Melting (i.e., disassociation) temperatures of PCR amplicons depend mainly on amplicon size and GC content, and can be used as an additional diagnostic tool to verify target sequence amplification (*35,39,40*). As shown in Figure 7.2, the average melting temperatures (T_m) for the amplicons produced with genomic and plasmid (pSZ16S) DNA of strain SZ were 83.6 ± 0.0 ºC and 83.6 ± 0.4 ºC, respectively. These data agree well with the estimated T_m of 85ºC calculated with the Oligonucleotide Properties Calculator (version 3.13; http://www.basic.northwestern.edu/biotools/oligocalc.html) for the 357 bp amplicon. Minimal amplification (i.e., below the quantification limit) was observed with triplicate samples of *G. sulfurreducens* and *G. metallireducens* genomic DNA, and no amplification was observed without template DNA. Some amplification (i.e., fluorescence) was observed with *G. thiogenes*, although the shape of the amplification plot was markedly different than the shape of the plots for strain SZ. In fact, the software available with the ABI 7500 Fast Real-Time PCR System failed to quantify samples of *G. thiogenes* due to a "noisy baseline." The melting curves for samples of *G. thiogenes*, with an average T_m of 70.5 ± 0.0 ºC, were also markedly different than the curves for strain SZ (Figure 7.2). This melting temperature does not agree with the T_m of 84ºC predicted for *G. thiogenes*. Similar melting curves and temperatures were obtained when 1:10 and 1:100 dilutions of the genomic DNA of

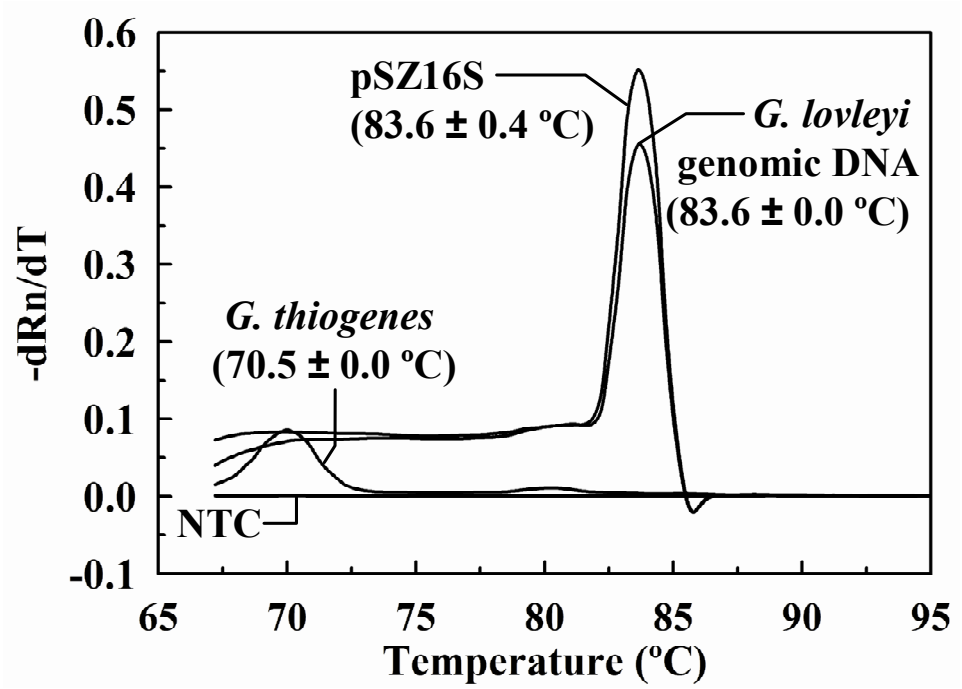

Figure 7.2 Melting curve analysis following qPCR for samples of *Geobacter lovleyi* strain SZ, *Geobacter thiogenes*, and no template controls (NTCs. For strain SZ, samples included genomic DNA or plasmid DNA with a 16S rRNA gene insert of strain SZ (pSZ16S). For *G. thiogenes*, the samples were genomic DNA. The NTCs did not contain template DNA. The melting curves represent averages of triplicate qPCR reactions for each sample, and average melting temperatures (T_m ± standard deviation) for each sample are listed in parentheses. –dRn/dT represents the negative derivate of the reported fluorescent signal (Rn) with respect to temperature (T). The T_m for the target amplicon is at the maximum rate of change (i.e., the greatest –dRn/dT) for each melting curve.

G. thiogenes were used as templates in qPCR. When qPCR products were analyzed on agarose gels, amplicons of the expected size (357 bp) were observed for strain SZ, but no amplicons were produced with *G. thiogenes* template DNA. Fluorescence was not observed with samples of strain SZ or *G. thiogenes* when either the forward (Geo195F) or reverse (Geo535R) primer was omitted from the PCR reaction. A possible explanation for the observed fluorescence with *G. thiogenes* is the formation of primer dimers, which typically have lower melting temperatures than PCR amplicons (*35*). Primer dimers are usually most pronounced in NTCs (*35*). As shown in Figure 2, a peak indicating primer dimers was not detected in the NTCs; similarly, the melting curves of *G. metallireducens* or *G. sulfurreducens* samples did not indicate the formation of primer dimers. These results suggest that the observed fluorescence with *G. thiogenes* was not caused by primer dimer formation. The mechanism(s) by which fluorescence was produced with *G. thiogenes* DNA during qPCR is unclear, but may be related to the few mismatches of primer Geo196F and perfect base pair matching of primer Geo535R with the 16S rRNA gene sequence of *G. thiogenes* (see Table 7.1). Although some amplification of *G. thiogenes* DNA occurred with the described qPCR approach, multiple lines of evidence (e.g., melting curves, amplification plots, noisy baselines) distinguished *G. lovleyi* strain SZ from *G. thiogenes* during qPCR analysis. Similar discrimination of closely related organisms by melting curve analysis has been described for distinguishing and identifying medically relevant microorganisms (summarized by Robinson et al. (*40*)). Hence, melting curve analysis may also be a useful tool to differentiate environmentally relevant bacteria with highly similar target sequences, potentially allowing for high-resolution discrimination (*39*).

Although the qPCR approach clearly identified false positive signals (i.e., fluorescence), *G. thiogenes* DNA influenced the quantification of strain SZ when DNAs from both isolates were present in the same sample (Table 7.2). In the absence of *G. thiogenes* DNA, the average estimate for the number of strain SZ cells per mL of a PCE-grown culture was $1.50 \pm 0.11 \times 10^8$. The estimated cell numbers increased linearly ($R^2 = 0.971$) in the presence of increasing concentrations of *G. thiogenes* DNA by up to 1.78 fold (Table 7.2). Ten-fold dilution of pure strain SZ template DNA did not have a significant effect on the cell number estimate (Table 7.2), indicating that the observed effect of *G. thiogenes* DNA was not due to dilution of strain SZ DNA. The increased estimate for strain SZ in the presence of *G. thiogenes* is likely related to the non-specific fluorescent signal produced with DNA of *G. thiogenes* (see above) that may have increased the overall fluorescence in the sample (i.e., fluorescence due to strain SZ plus fluorescence due to *G. thiogenes*). The melting curves following qPCR with mixtures of strain SZ and *G. thiogenes* DNA did not contain the characteristic peak for *G. thiogenes* (~70.5ºC, see Figure 7.2) but only the characteristic peak (average T_m of 83.9 ± 0.3 ºC) for strain SZ. These results suggest that qPCR overestimates the abundance of strain SZ in the presence of *G. thiogenes* DNA and that melting curve analysis cannot identify when the overestimation occurs (i.e., when *G. thiogenes* and strain SZ DNA occur concomitantly in a sample). Hence, the development of qPCR protocols and other quantitative, nucleic acid-based tools should not only evaluate the specificity of the approach but also explore the effects of non-target DNA, including DNA with high sequence similarity to the target DNA sequence, on the accuracy of quantification (*41*).

7.4.2 Detection of Strain SZ in Chlorinated Ethene-Dechlorinating Enrichment Cultures and at Chlorinated Ethene-Contaminated Sites

Strain SZ-like organisms were detected with the Geo196F/Geo999R primer pair in the PCE-to-ethene dechlorinating Hydrite enrichment culture (direct PCR, Figure 7.3). With acetate provided as electron donor, *cis*-DCE accumulated in the Hydrite culture, whereas complete reductive dechlorination to ethene occurred when the cultures were amended with hydrogen (*26*). This dechlorination pattern is consistent with dechlorinator physiology: strain SZ couples either acetate or hydrogen oxidation to the reduction of PCE and TCE to *cis*-DCE (*11*) and *Dehalococcoides* sp. strain GT, which was isolated from the Hydrite culture (*42*), requires hydrogen as electron donor to drive the process to completion (i.e., ethene formation). No amplification with the Geo196F/Geo999R primer pair was observed with DNA samples from three other PCE-to-ethene dechlorinating mixed cultures in either direct (Figure 7.3) or nested PCR. Strain SZ-like amplicons were obtained from the KB-1 bioaugmentation consortium (direct PCR, Figure 3), from which a cloned 16S rRNA gene sequence with high similarity to that of strain SZ was obtained (*28,29*). Recent studies have demonstrated growth of the SZ-like organism in the KB-1 consortium during PCE and TCE dechlorination to *cis*-DCE (*43*). Strain SZ-like organisms were not detected in the bioaugmentation consortium Bio-Dechlor INOCULUM via direct (Figure 7.3) or nested PCR. Both bioaugmentation inocula have been successfully used to promote *in situ* reductive dechlorination of chlorinated ethenes (*44,45*). Strain SZ-like organisms were also detected in groundwater samples from a TCE-impacted aquifer near Milledgeville, Georgia (nested PCR, Figure 7.3). Dechlorination of TCE to stoichiometric amounts of *cis*-DCE was observed in microcosms and occurred *in situ* after biostimulation (*46*), suggesting that strain SZ-like

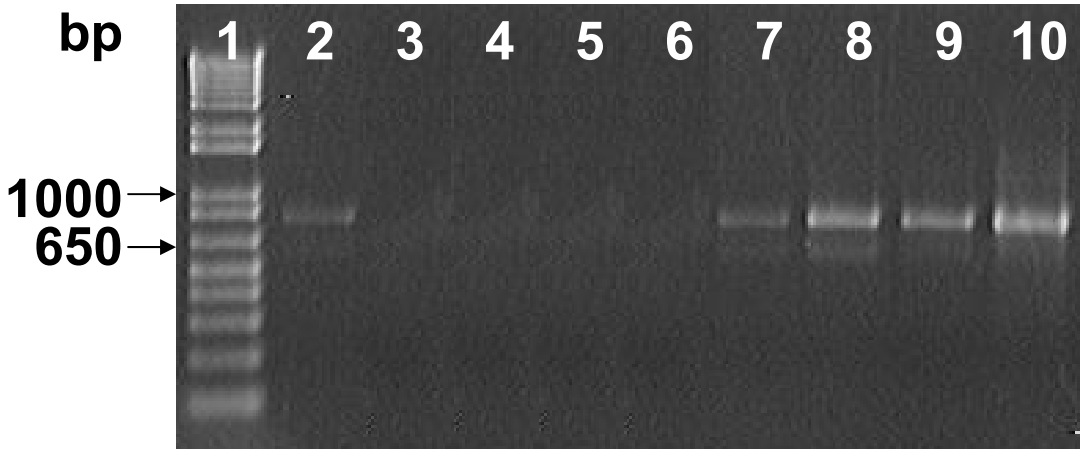

Figure 7.3 Detection of strain SZ in samples from chlorinated ethene-dechlorinating enrichment cultures and in groundwater samples from chlorinated ethene-contaminated sites with the 16S rRNA gene-targeted primer pair Geo196F/Geo999R: direct PCR (lanes 2 to 7); nested PCR (lanes 8 and 9). Lane 1, DNA size marker (Invitrogen); lane 2, Hydrite culture; lane 3, Minerva culture; lane 4, FMC culture; lane 5, Kalamazoo culture; lane 6, Bio-Dechlor INOCULUM; lane 7, KB-1 consortium; lane 8 and 9, groundwater samples collected from two monitoring wells at a chlorinated ethene-contaminated site in Georgia; lane 10, strain SZ.

organisms were stimulated and played a role in the observed dechlorination. No SZ-specific signals were obtained with DNA extracted from TCE-contaminated Ft. Lewis soil. However, amplification with the Geo196F/Geo999R primer pair occurred in nested PCR with DNA obtained from a TCE- and lactate-amended enrichment culture derived from the Ft. Lewis soil, and qPCR estimated $4.34 \pm 0.96 \times 10^6$ strain SZ-like cells per mL of culture. Additionally, three 16S rRNA gene sequences with 96-99% similarity to that of strain SZ were detected in a clone library constructed with DNA extracted from the Ft. Lewis culture (*27*).

Detection of strain SZ-like organisms in chlorinated ethene-dechlorinating enrichment cultures, the KB-1 bioaugmentation consortium, and in environmental samples from chlorinated ethene-impacted aquifers suggests that dechlorinating *Geobacter* spp. may be relevant contributors to chlorinated ethene detoxification. This hypothesis is corroborated by the results presented in Chapter 5 and previous detection of SZ-like 16S rRNA gene sequences in PCE-to-ethene dechlorinating mixed cultures and contaminated aquifers (*28,29,43,47-49*). Apparently, chlorinated ethene dechlorinating *Geobacter* species are not rare in the environment, thus expanding the understanding of the physiological versatility of the *Geobacteraceae*. Additional dechlorinating *Geobacter* isolates are needed to refine the parameters delineating the putative dechlorinating clade within the *Geobacteraceae* (*11*).

7.4.3 Detection of strain SZ at the Oak Ridge IFC site

Strain SZ-like organisms were detected in community genomic DNA extracted from 13 of 22 Oak Ridge IFC site groundwater samples. As shown in Figure 7.4A,

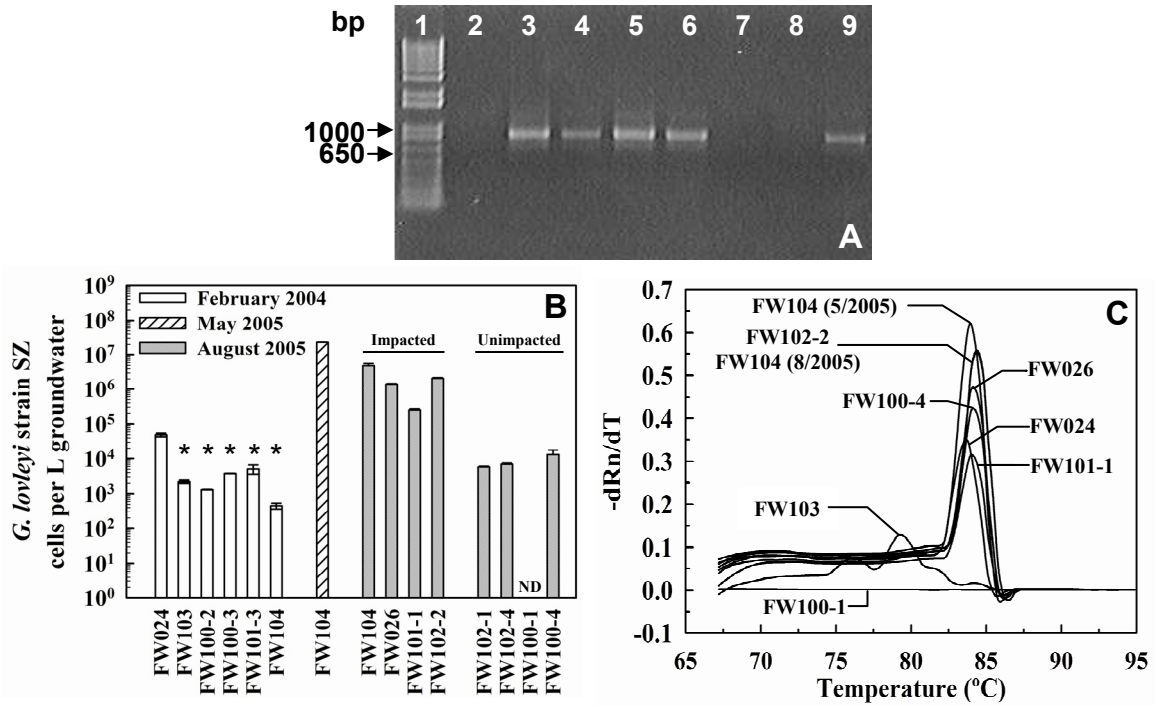

Figure 7.4 Detection of strain SZ at the uranium-contaminated Oak Ridge Integrated Field-Scale Subsurface Research Challenge (IFC) site. (A) Detection of strain SZ-like organisms via nested PCR in Oak Ridge IFC site groundwater taken from regions impacted by biostimulation (wells FW026 [lane 3], FW029 [lane 4], FW101-2 [lane 5], FW102-3 [lane 6]) or not impacted by biostimulation (wells FW016 [lane 2], FW106 [lane 7], and TPB16 [lane 8]). Lane 1 is the DNA size marker (Invitrogen), and lane 9 is genomic DNA of strain SZ. (B) Average *G. lovleyi* strain SZ cell numbers per liter of Oak Ridge IFC site groundwater in a pilot-scale uranium bioreduction demonstration plot. Samples were taken approximately 1 month after the initial ethanol amendment (February 2004) and in May and August 2005, after 1-1.5 years of periodic ethanol biostimulation. The samples from August 2005 came from aquifer regions impacted or not impacted by biostimulation, as indicated. Error bars indicate standard deviations of triplicate qPCR reactions and are not shown when they are too small to depict. The asterisks (*) indicate that the cell number estimates were extrapolated values outside the range of accurate quantification and that the melting curves did not contain the characteristic peak for strain SZ (see text for details). ND; fluorescence was not detected. (C) Melting curves following qPCR for representative samples from the Oak Ridge IFC site. The melting curves represent averages of triplicate qPCR reactions for each sample. For an explanation of –dRn/dT see the caption of Figure 7.2.

specific amplicons were obtained with direct and nested PCR from samples of wells FW026, FW101-2, and FW102-3 located within the Area 3 biostimulated zone (*5,31*). Nested PCR yielded an additional positive signal with DNA collected from well FW029 (Area 1), which was also influenced by biostimulation (Figure 7.4A) (*30*). Sequence analysis of at least 350 bp of all amplicons confirmed sequence identity with the 16S rRNA gene sequence of strain SZ. No amplification was observed through direct or nested PCR (see Figure 7.4A) with samples from wells FW016 (Area 1), TPB16 (Area 2), and FW106 (Area 3), which are located in areas that received no biostimulation treatment. The geochemistry (e.g., low pH and high nitrate) of well FW106 is similar to that of the Area 3 biostimulated zone prior to conditioning and periodic ethanol amendment (*5,31*). Detection of strain SZ-like organisms in samples from the biostimulated plot, but not from well FW106, indicates that strain SZ responded to biostimulation at the Oak Ridge IFC.

As depicted in Figure 7.4B, strain SZ-like organisms could also be enumerated in various groundwater samples collected within the uranium bioreduction plot at the Oak Ridge IFC site (*5,31*). In February 2004, approximately one month after the initial amendment of ethanol to the inner recirculation loop (see Section 7.3 for a brief description of the inner and outer recirculation loops), the number of strain SZ cells was less than 5×10^4 per liter groundwater (Figure 7.4B). Minimal amplification (i.e., fluorescence) was observed in most of the samples from February 2004 (see asterisks [*] in Figure 7.4B), and cell number estimates for these samples were extrapolated from the linear qPCR standard curve. These estimates must be interpreted cautiously since the fluorescent signals were below the limit for accurate quantification (~30 16S rRNA gene

copies per μL of template DNA; see above). The melting curves for these samples did not have the characteristic strain SZ peak, but instead several smaller peaks were visible (Figure 7.4C). All samples that could be accurately quantified had melting curves with the characteristic peak for strain SZ (Figure 7.4C). These results demonstrate that melting curve analysis was useful to distinguish nonspecific amplification and identify false positive signals with environmental samples. Samples taken following biostimulation indicated substantial growth of strain SZ-like organisms (Figure 7.4B). For example, cell numbers in samples from the inner recirculation loop injection well (FW104) increased by over three orders-of-magnitude from February 2004 to May and August 2005 (Figure 7.4B). The samples from May and August 2005 were taken approximately 1-1.5 years after the onset of periodic ethanol addition to the inner recirculation loop, indicating that strain SZ persisted at high levels in the biostimulated zone throughout the biostimulation period. In August 2005, strain SZ cell numbers ranged from 10^5 - 10^6 per liter of groundwater in regions impacted by biostimulation (Figure 7.4B). In contrast, the number of SZ cells was significantly lower in samples taken at the same time from areas not impacted by biostimulation (Figure 7.4B).

These observations indicate that strain SZ-like organisms were present at the Oak Ridge IFC site and, importantly, responded to biostimulation. The addition of ethanol and/or adjustment of other geochemical parameters (e.g., pH, nitrate removal (*5,31*)) stimulated growth of strain SZ-like organisms. Although strain SZ cannot utilize ethanol as a direct electron donor, ethanol fermentation yields hydrogen and acetate, electron donors used by strain SZ (*11*). There is ample evidence that microbes indigenous to the Oak Ridge IFC site produce acetate when stimulated by ethanol: acetate accumulated in

(i) ethanol-amended microcosms containing sediment from well FW104 of the Oak Ridge IFC site (*50*), (ii) in an ethanol-amended soil column containing sediment from FW104 (*51*), and (iii) in portions of the Area 3 biostimulated zone following ethanol addition (*31*). In a sulfate-reducing and U(VI)-reducing enrichment culture derived from the soil column and dominated by *Desulfovibrio*-like species, ethanol addition led to the accumulation of acetate in molar excess of the added amount, indicating homoacetogenenic activity (*52*). Hydrogen also accumulated intermittently. When the enrichment was transferred without sulfate, *Clostridium*-like bacteria became dominant while acetate and hydrogen accumulated. These observations indicate that several processes produce acetate (and hydrogen) from ethanol in the Oak Ridge IFC site subsurface and that strain SZ could potentially partner with different types of bacteria at the site to reduce U(VI). Previous work has demonstrated that *G. lovleyi* strain SZ reduces U(VI) and PCE simultaneously (*11*), and hence, strain SZ is a likely contributor to *in situ* bioremediation at the mixed waste Oak Ridge IFC site (*4,5*). The identification of key organisms contributing to contaminant detoxification (e.g., U(VI) reduction and reductive dechlorination) represents a targeted approach for bioremediation monitoring. Although comprehensive information of the microbial community composition and dynamics at bioremediation sites is desirable, the application of targeted monitoring approaches to delineate and quantify the contributions of select organisms may produce tangible results in the short term. Monitoring key contributors such as strain SZ-like organisms and other U(VI)-reducing bacteria (e.g., *Anaeromyxobacter* spp. and other metal reducers) to U(VI) bioreduction at the Oak Ridge IFC site and other uranium-

impacted sites may provide sufficient information for productive implementation of bioremediation technologies.

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CHAPTER 8

OXYGEN EFFECT ON *DEHALOCOCCOIDES* VIABILITY AND QUANTIFICATION

Reproduced in part with permission from Amos, B. K.; Ritalahti, K.; Cruz-Garcia , C.; Padilla-Crespo, E.; and Löffler, F. E. Oxygen effect on *Dehalococcoides* viability and quantification. In preparation.

8.1 Abstract

Strict anaerobic *Dehalococcoides* spp. play a critical role in detoxification of chlorinated contaminants. During bioremediation of contaminated aquifers, *Dehalococcoides* cells may be exposed to oxygen, but the effects of oxygen on *Dehalococcoides* viability and biomarker quantification have not been evaluated. Batch culture experiments to assess the influence of oxygen (10% [vol/vol]) on *Dehalococcoides* activity were performed with a chlorinated ethene-dechlorinating consortium (Bio-Dechlor INOCULUM [BDI]) that contains multiple *Dehalococcoides* strains. In oxygen-amended cultures, trichloroethene (TCE) dechlorination was initially inhibited before subsequent conversion of TCE to vinyl chloride (VC) and traces of ethene. Dechlorination was completely inhibited in oxygen-amended, VC-fed cultures. Reduced (i.e., oxygen-free) control cultures, however, completely dechlorinated TCE or VC to ethene. To test the reversibility of oxygen inhibition, biomass from both the control and oxygen-amended cultures was collected and transferred to oxygen-free medium. TCE-to-ethene and VC-to-ethene dechlorination activity occurred in the control

cultures. In contrast, the TCE-fed reversibility cultures only dechlorinated TCE to VC with slow (cometabolic) accumulation of ethene. Likewise, the majority (7 of 8) of the VC-fed reversibility cultures was unable to dechlorinate VC to ethene (262-day incubation). VC dechlorination to ethene took >160 days to reach completion in the remaining VC-fed reversibility culture. These results suggest strain specific differences in the ability of *Dehalococcoides* cells to remain viable following exposure to oxygen. Although quantitative real-time PCR (qPCR) analysis indicated a ~1-1.5 order-of-magnitude decrease in the number of *Dehalococcoides* biomarker genes (i.e., 16S rRNA and reductive dehalogenase [RDase] genes [*tceA, vcrA, bvcA*]) in the oxygen-amended cultures, qPCR analysis easily detected the irreversibly-inhibited (nonviable) *Dehalococcoides* cells. *Dehalococcoides* RNA biomarkers (i.e., gene transcripts) were also detected in both the oxygen-amended and control cultures. RDase gene transcription correlated to available substrates (e.g., TCE or VC) in the control cultures but did not correlate to activity or substrate availability in the oxygen-amended cultures. In many cases, RNA biomarker analysis could not distinguish between inactive (i.e., oxygen-amended) and active (i.e., control) cultures. These results suggest that current methodologies to detect *Dehalococcoides* DNA and RNA biomarkers may have limitations for inferring cell viability and activity. New molecular tools are needed to complement exiting technologies to improve the application of biomarker analysis in bioremediation monitoring and assessment.

8.2 Introduction

Chlorinated ethenes are common groundwater pollutants with stringent regulatory standards. The current maximum contaminant levels (MCLs) for tetrachloroethene (PCE), trichloroethene (TCE), *cis*-1,2-dichloroethene (*cis*-DCE), *trans*-1,2-dichloroethene (*trans*-DCE), and vinyl chloride (VC) are 5 ppb, 5 ppb, 70 ppb, 100 ppb, and 2 ppb, respectively (US EPA; http://www.epa.gov/safewater/contaminants). These low concentration standards require corrective action(s) at many sites impacted with chlorinated ethenes. Traditionally, pump-and-treat systems have been deployed to prevent expansion of contaminant plumes at numerous sites, but this technology is inefficient, has high operating costs, and must be maintained for years to decades. The need for affordable remedial technologies has driven substantial research investment to identify alternative remedial actions that are productive and yield short-term benefits. Remarkable progress has been made in understanding the microbiology involved in detoxification of chlorinated ethenes (*1*), and bioremediation has emerged as a viable remediation strategy (*2*). A milestone for the success of chlorinated ethene bioremediation was the identification of *Dehalococcoides* spp. as the key catalysts involved in reductive dechlorination of DCEs and VC to innocuous products (i.e., ethene and inorganic chloride) (*3*-*6*). Laboratory and field-scale investigations demonstrated a cause-and-effect relationship between the presence of *Dehalococcoides* spp. and ethene formation (*7*-*10*). Hence, assessment of chlorinated ethene-impacted sites where bioremediation is considered a treatment option includes one key objective: are *Dehalococcoides* spp. present or not? To address this question, *Dehalococcoides*-specific

PCR-based tools are applied to DNA extracted from site samples (e.g., groundwater). The current site assessment and monitoring tools target *Dehalococcoides* 16S rRNA and reductive dehalogenase (RDase) genes (i.e., *tceA*, *bvcA* and *vcrA*), which are implicated in chlorinated ethene reductive dechlorination (*11-13*). PCR-based approaches for sensitive, specific detection and quantification of *Dehalococcoides* biomarker genes have been designed (*7,8,14-20*) and are offered commercially (e.g., Microbial Insights [http://www.microbe.com], SiREM [http://www.siremlab.com]).

Dehalococcoides spp. are native to many sites contaminated with chlorinated ethenes, but the dechlorinating bacteria are often patchily distributed throughout the aquifer and/or are present in very low numbers (*9,10*). At sites where native *Dehalococcoides* spp. occur, the lack of substrates and/or suitable redox conditions often limit (i.e., control) reductive dechlorination and detoxification. To overcome the nutritional bottleneck(s), biostimulation with organic (e.g., lactate) and inorganic (i.e., N and P) substrates have been successfully implemented at the field scale (*9*). Although biostimulation has been employed productively at several sites, this approach may be insufficient to sustain desirable dechlorination rates and only works at sites that have native *Dehalococcoides* spp. capable of efficient ethene formation. As an alternative approach, bioaugmentation with *Dehalococcoides* spp.-containing consortia has been implemented at numerous sites (*9,10,21,22*), and bioaugmentation inocula are commercially available (e.g., SiREM, http://www.siremlab.com; Shaw, http://www.shawgrp.com/home).

To accompany these bioremediation field efforts, detailed laboratory studies with *Dehalococcoides* pure and mixed cultures have been performed to elucidate the

organisms' physiology and nutritional requirements. *Dehalococcoides* spp. are very difficult to grow and maintain in pure culture (*3,5*). The reasons for the intricate growth of *Dehalococcoides* spp. in pure culture is unclear but may be due to unknown nutritional requirements and/or sensitivity to oxygen (*3,5*). The effects of oxygen on *Dehalococcoides* viability have not been thoroughly explored, but several studies reported that brief exposure of *Dehalococcoides* spp. to air and/or oxygen completely and irreversibly inhibited dechlorination (*8,23,24*). Therefore, bioaugmentation with *Dehalococcoides* containing consortia include techniques that reduce exposure of the bioaugmentation culture to air (*25*); however, for practical reasons this is difficult to achieve at many contaminanted sites. Obviously, oxygen exposure during inoculum transport to the contaminanted site and/or delivery to the subsurface may have a profound impact on the success of the biological remedy. Additionally, oxygen may be delivered to aquifers and potentially contact subsurface *Dehalococcoides* organisms during infiltration of oxygenated groundwater (e.g., rain events) or during injection of remedial fluids (e.g., electron donor). Hence, the aim of this study was to explore the effects of oxygen on *Dehalococcoides* viability and dechlorination performance in more detail, and evaluate if the current PCR-based tools are useful to detect oxygen exposure and distinguish viable, dechlorinating from inactive, oxygen-exposed cells. To this end, we also explored the use of RNA rather than DNA biomarkers as useful indicators of oxygen exposure. Information on *Dehalococcoides* spp. survivability (i.e., resistance and resilience) following oxygen exposure and knowledge of the resolution of the current molecular tools to detect oxygen exposure has practical relevance for bioremediation field applications.

8.3 Materials and Methods

8.3.1 Chemicals

TCE (≥99.5%) was obtained from Sigma-Aldrich Co. (St. Louis, Missouri). *cis*-DCE (99.9%) and *trans*-DCE (99.9%) were purchased from Supelco Co. (Bellefonte, Pennsylvania). Gaseous VC (≥99.5%) was purchased from Fluka Chemical Corp. (Ronkonkoma, New York), and gaseous ethene (99.5%) was obtained from Scott Specialty Gases (Durham, North Carolina). All other chemicals used were reagent grade or better unless otherwise specified.

8.3.2 Culture, Medium Preparation, and Growth Conditions

Bio-Dechlor INOCULUM (BDI), a PCE-to-ethene dechlorinating consortium, was used in this study. The BDI consortium has been successfully used in bioaugmentation field applications (*21*) and contains at least three *Dehalococcoides* strains: strains FL2, GT, and BAV1 (*15*). Strain FL2 metabolically dechlorinates TCE to VC and cometabolically transforms VC to ethene (*4*). Strain GT metabolically dechlorinates TCE to ethene (*6*), while strain BAV1 metabolically dechlorinates *cis*-DCE-to-ethene (*3*). These *Dehalococcoides* strains can be tracked via quantitative real-time PCR (qPCR; (*15*)) by targeting specific RDase genes: the TCE-to-VC RDase gene (*tceA*) of strain FL2 (*11*), the *cis*-DCE-to-ethene RDase gene (*vcrA*) of strain GT (*6,13*), and the putative VC-to-ethene RDase gene (*bvcA*) of strain BAV1 (*12*). BDI also contains a PCE-to-*cis*-DCE dechlorinating *Dehalobacter* species (*24,26*). Reduced anaerobic mineral salts medium was prepared as described (see *Supporting Information*

of reference (*24*)), except the concentration of Na_2S x $9H_2O$ was 0.1 mM. The medium contained resazurin (1 μM; 0.25 mg/L) as a redox indicator (*27-30*). Resazurin, which can be reoxidized by oxygen (*27*), is only useful as a redox indicator in a narrow range of redox potentials (-51 mV ± 60 mV, pH 7 (*29*)). Solutions containing resazurin are bright pink at redox potentials above +10 mV, colorless at redox potentials below -110 mV, and various intensities of pink at redox potentials between -110 mV and +10 mV. The BDI consortium was grown in 160-mL (nominal volume; Wheaton Co, Millville, New Jersey) glass serum bottles containing 100 mL ± 1 mL of medium and a N_2/CO_2 (80%/20% [vol/vol]) headspace (unless otherwise indicated). Bottles were sealed with Teflon-lined, gray butyl-rubber septa (West Pharmaceuticals, Lionville, Pennsylvania) held in place with aluminum crimp caps (Wheaton). Lactate (5 mM) and hydrogen (10 mL) served as electron donors. Lactate was added to individual bottles from a sterile, anoxic stock solution, while sterile hydrogen gas was added via syringe and 30 gauge needle. TCE (4 μL of neat liquid) or VC (3 mL sterile gas) were provided as electron acceptors. TCE was added with a 5-μL gastight syringe (model 95 with a reproducibility [Chaney] adapter; Hamilton Co., Reno, Nevada); VC was added via 3-mL disposable syringe. Triplicate or duplicate cultures were incubated statically in upside down position at room temperature.

8.3.3 Oxygen Exposure

Triplicate bottles containing fresh medium were incubated in the presence of oxygen. Sterile oxygen gas was amended to individual bottles via syringe and 30 gauge needle at an initial amount of 10% (vol/vol) of the headspace. Triplicate control cultures

did not receive oxygen but instead were amended with equal volumes of nitrogen gas. Each vial was allowed to equilibrate for 2 days after addition of oxygen (or nitrogen) and VC or TCE before inoculation with 5% (vol/vol) from an actively dechlorinating ~10-L stock culture of BDI, which received periodic additions of TCE as electron acceptor and lactate as electron donor. Before inoculation to individual bottles, two aliquots (100 mL each) of BDI from the stock culture were dispensed into sterile, N_2-flushed serum bottles. Filter-sterilized streams of N_2/CO_2 (80%/20% [vol/vol]) were bubbled through each cell suspension for 15 minutes to remove residual chlorinated ethenes. Three 5-mL samples of each cell suspension were collected for qPCR and reverse transcription qPCR (RT-qPCR) analyses before distribution of the inoculum to the VC-fed or TCE-fed experimental bottles. The oxygen exposure experiment was repeated in an independent experiment with duplicate, VC-fed cultures and the following modifications: the inoculum size was increased to 15% (vol/vol) and oxygen was initially provided at 3.5% (nominal, vol/vol) of the headspace. In the independent experiment, subsequent amendments of oxygen on Days 10 and 15 of the 21-day incubation raised the oxygen concentration to 21 and 28% (nominal, vol/vol) of the headspace, respectively. Liquid samples were taken periodically from both sets of experiments for DNA and/or RNA extraction and subsequent analysis via qPCR and/or RT-qPCR as described below.

8.3.4 Reversibility Experiments

To determine if the effect of oxygen exposure on *Dehalococcoides* organisms present in BDI was reversible, aqueous 1 mL samples from triplicate VC- or TCE-fed, oxygen-amended cultures were transferred on Day 30 of the incubation to triplicate

vessels containing reduced, oxygen-free medium equilibrated with VC or TCE, respectively. Aqueous samples (1 mL) were also transferred to triplicate vessels containing reduced, oxygen-free medium from triplicate BDI cultures incubated without oxygen (positive controls). Due to concern that the transferred 1-mL samples contained sufficient amounts of oxygen to inhibit subsequent dechlorination activity in the fresh medium, separate reversibility experiments were also conducted with washed cell suspensions as described by Amos et al. (*24*) to ensure complete removal of oxygen from the transferred culture suspensions. Briefly, biomass was collected by centrifugation (4,300 x g, 30 minutes) in an anoxic glove box (Coy Laboratory Products, Ann Arbor, Michigan) from 5 mL of triplicate VC- or TCE-fed, oxygen-amended or positive control cultures on Day 30 of the incubation. The resulting cell pellets were washed once with reduced (i.e., oxygen-free) medium and suspended in 1 mL of the reduced medium. The washed cell suspensions served as inocula to triplicate (duplicate for the VC-fed, positive control cultures) vessels containing reduced, oxygen-free medium amended with VC or TCE, respectively. In the independent, repeated oxygen-exposure experiment, reversibility experiments were performed with washed cell suspensions collected from 15 mL of culture on Day 21. The washed cell suspensions served as inocula for duplicate vessels containing reduced, oxygen-free medium amended with VC.

8.3.5 DNA and RNA Extraction

Biomass was collected periodically from aqueous samples of culture fluid by centrifugation at 16,000 x g for 10 minutes. The supernatant was decanted, and the biomass from five 1-mL samples of culture suspension was collected in the same tube.

The resulting pellet was slowly suspended by pipetting in 500 µL of RNAprotect Bacteria Reagent (Qiagen, Valencia, California) to stabilize and protect RNA *in vivo*. The sample was homogenized by vortexing, incubated at room temperature for 5 minutes, and centrifuged at 16,000 x g for 10 minutes. The supernatant was removed, and genomic DNA and total RNA were extracted with the AllPrep DNA/RNA Mini Kit (Qiagen) from frozen cell pellets. During nucleic acid extraction, the cell pellets were suspended in 250 µL Tris-EDTA (TE) buffer (RNase-free, pH 8; Ambion, Austin, Texas) containing 15 mg/mL lysozyme (Sigma). Each sample then received 1 µL of 10% SDS solution (20% RNase-free SDS stock [Ambion] diluted with diethyl pyrocarbonate- [DEPC-] treated water) and 600 µL RTL lysis buffer (supplied with AllPrep DNA/RNA Mini Kit) containing 0.14 M β-mercaptoethanol (Sigma). Select samples (4 of 30) also received an amendment of control RNA to estimate RNA recovery (see below). All samples were then vortexed for 5 minutes before following the remainder of the manufacturer's protocol. DNA was obtained in a final volume of 100 µL buffer EB (provided with the AllPrep DNA/RNA Mini Kit) and stored at -20°C until qPCR analysis. RNA was obtained in a final volume of 100 µL RNase-free water, amended with 1 µL of RNaseOUT Ribonuclease Inhibitor (Invitrogen, Carlsbad, CA), and stored at -80°C until further processing.

In the independent experiment with VC-fed, oxygen-amended cultures, biomass was collected periodically from 10 mL of culture fluid by centrifugation as described (*24*). The cell pellet was stored at -20°C until genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, California) following the modifications

described by Ritalahti et al. (*15*). DNA was obtained in a final volume of 200 µL buffer AE (provided with the QIAamp DNA Mini Kit) and stored at -20°C until qPCR analysis.

8.3.6 RNA Purification and Reverse Transcription

Extracted RNA was DNase treated with the TURBO DNA-*free* kit (Ambion) according to the manufacturer's recommendations, to remove contaminating DNA. After the DNase treatment, RNA was purified by successive extractions with phenol, phenol:chloroform:isoamyl alcohol (25:24:1 vol/vol/vol), and chloroform:isoamyl alcohol (24:1 vol/vol) as described (*31*). RNA was recovered by ethanol precipitation with 0.3 M sodium acetate as described (*31*). The precipitated RNA was dissolved in 20 µL RNase-free water, amended with 1 µL of RNaseOUT Ribonuclease Inhibitor (Invitrogen), and stored at -80ºC until use. Removal of contaminating DNA was confirmed via PCR with universal bacterial 16S rRNA gene-targeted primers (*14*).

Reverse transcription was performed with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Before reverse transcription, the volumes of the RNA samples were reduced to <8 µL using a SC210A SpeedVac Plus (ThermoSavant, Milford, Massachusetts) for 30-40 minutes at room temperature. Subsequently, each reverse transcription reaction consisted of 1 µL random hexamer primers (50 ng/µL; provided with the SuperScript kit), 1 µL 10 mM dNTP mix (provided with the SuperScript kit), the RNA sample, and DEPC-treated water to bring the final reaction volume to 10 µL. Reverse transcription then proceeded according to the manufacturer's protocol. cDNA was obtained in a final volume of 21 µL and stored at -20°C until qPCR analysis.

8.3.7 Quantification of RNA Recovery

Quantification of RNA recovery during RNA extraction, purification, and conversion to cDNA was performed as described by Johnson et al. (*19*). Briefly, select cell pellets (4 of 30) received 2 x 10^{11} transcripts of Luciferase Control RNA (Promega, Madison, WI) as mentioned above (Section 8.3.5). The number of transcripts added to each sample was estimated from the RNA concentration (100 ng/µL; a 1:10 dilution of the purchased 1000 ng/µL stock), assuming an average molecular weight of 330 for a nucleotide of single-stranded RNA and a transcript size of 1.8 kb (*15,19*). RNA recovery was determined by dividing the total number of luciferase transcripts detected via qPCR analysis in the cDNA by the total number of transcripts added to each cell pellet. The average luciferase transcript recovery was 9.8 ± 0.9 % (n = 4; data not shown); this recovery is comparable to the recovery reported by Johnson et al. (*19*) for another chlorinated ethene-dechlorinating consortium. The transcript numbers reported in the text for the *Dehalococcoides* 16S rRNA, *tceA*, *vcrA*, and *bvcA* genes were adjusted to reflect the determined RNA recovery (*19*).

8.3.8 Quantitative Real-Time PCR (qPCR) Analysis

TaqMan-based qPCR analysis of DNA and cDNA was used to quantify the number of *Dehalococcoides* 16S rRNA, *tceA*, *vcrA*, and *bvcA* gene copies and transcript copies as described (*15*). qPCR analysis to quantify the luciferase control RNA utilized a TaqMan-based approach with the primers and probe designed by Johnson et al. (*19*). The probe (Integrated DNA Technologies, Coralville, Iowa) was modified to include 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE NHS Ester) as the reporter

fluorophore on the 5' end and a black hole quencher (BHQ-1) on the 3' end. Each well of a MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems, Foster, California) contained 1x TaqMan universal PCR master mix (ABI), 300 nM probe, 300 nM of each primer, and 2 µL of template DNA or cDNA in a total reaction volume of 20 µL. qPCR analysis was carried out in an ABI 7500 Fast Real-Time PCR System with cycle parameters as follows: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 58°C using the standard 7500 operating mode. Standard curves were generated for the *Dehalococcoides* 16S rRNA, *tceA*, *vcrA*, and *bvcA* genes as described (*15*). For the luciferase standard curve, luciferase control RNA transcripts were reverse transcribed as described above. The luciferase cDNA was quantified by spectrophotometry (*15*), and 10 ng of the luciferase cDNA was serially diluted (10-fold) for use as standards. The number of luciferase cDNA transcripts in each standard was estimated from the cDNA concentration and the size of the cDNA fragment. The terms "gene copies" and "cell numbers" are per mL of culture fluid and are used interchangeably because the known *Dehalococcoides* organisms contain one 16S rRNA gene copy per genome (*32,33*) and one target reductive dehalogenase gene copy per cell (*15*). Transcript copy numbers are reported on a per cell basis (i.e., 16S rRNA gene transcript copy numbers divided by 16S rRNA gene copies; *tceA* gene transcript copy numbers divided by *tceA* gene copies; and *vcrA* gene transcript copy numbers divided by *vcrA* gene copies).

8.3.9 Analytical Methods

Aqueous phase (1 mL) samples were collected periodically for chlorinated ethene and ethene quantification by gas chromatography. Aqueous phase samples were analyzed with a Hewlett-Packard (HP) 7694 headspace autosampler connected to a HP 6890 gas chromatograph (GC) equipped with a HP-624 column (60 m by 0.32 mm; film thickness, 1.8 μm nominal) and a flame ionization detector (FID) as described (*24,34*). A Waters High Performance Liquid Chromatography (HPLC) system (Waters, Corp., Milford, Massachusetts) equipped with a Waters 2487 dual-wavelength absorbance detector set at 210 nm and a Waters 717 plus autosampler was used to analyze organic acids as described (*8*).

8.4 Results

8.4.1 Effect of Oxygen on *Dehalococcoides* Activity and Cell Numbers

The presence of oxygen negatively affected the ability of the BDI consortium to dechlorinate VC (Figure 8.1A) and TCE (Figure 8.2A). As shown in Figure 8.1, oxygen (initially at 10% [vol/vol] of the headspace) completely inhibited VC dechlorination, whereas control cultures without oxygen produced stoichiometric amounts of ethene. In the oxygen-amended cultures, ethene formation did not occur even after extended incubation (89 days). The medium was initially pink in the oxygen-amended vials, indicating that the redox indicator resazurin was oxidized. After Day 16, the medium started to turn colorless (i.e., resazurin reduced); by Day 30, the medium was colorless and slightly turbid. Lactate was completely fermented to acetate (2.57 ± 0.03 mM,

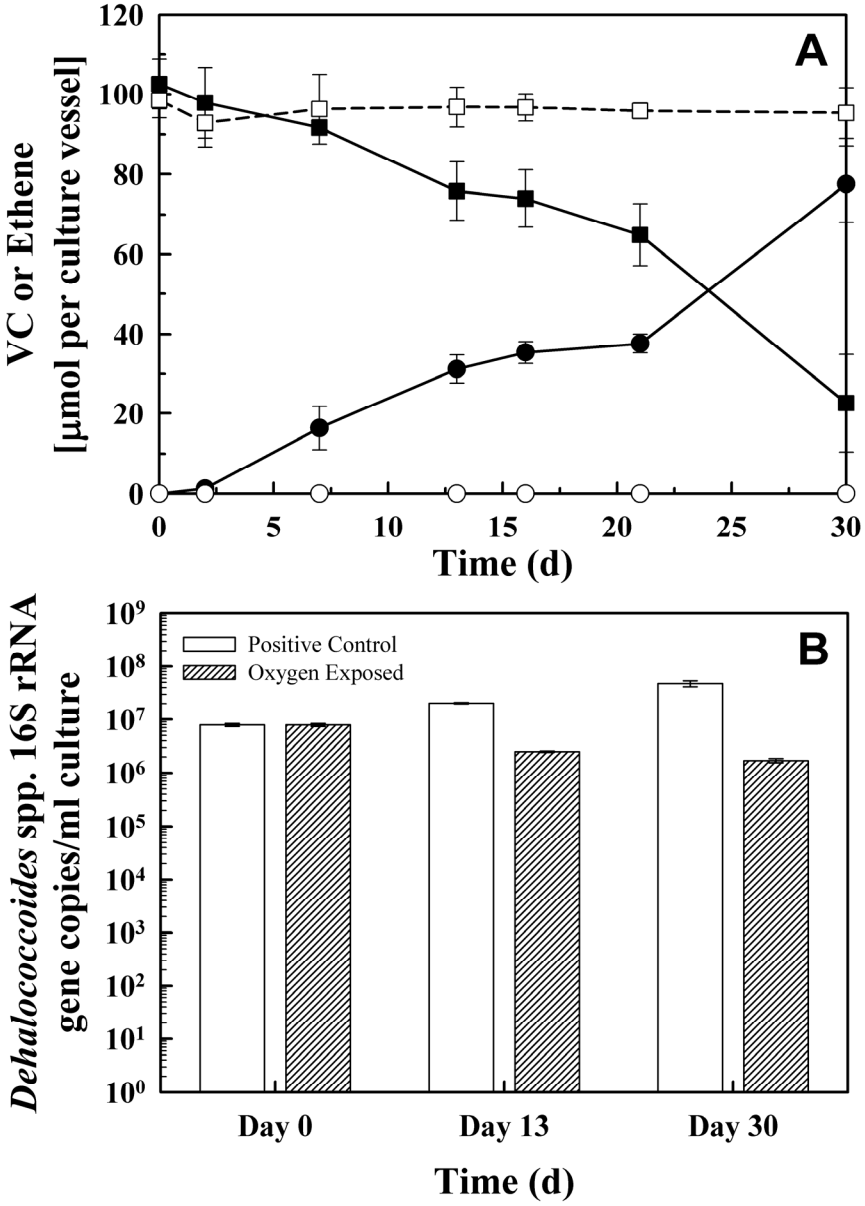

Figure 8.1 Dechlorination performance and qPCR analysis of the VC-fed BDI consortium. (A) Dechlorination performance of the VC-fed BDI consortium in the oxygen-amended (open symbols, dashed lines) or positive control (closed symbols, solid lines) cultures. Symbols: ■, VC; ●, ethene. (B) qPCR analysis of *Dehalococcoides* spp. 16S rRNA gene copy numbers in the oxygen-amended and positive control cultures. All data points represent average values from triplicate cultures, and error bars represent one standard deviation. When error bars are not visible, they are smaller than and therefore hidden behind the data symbols.

propionate (2.7 ± 0.2 mM), and formate (1.1 ± 0.3 mM) in the oxygen-amended cultures, indicating that fermentation yielded adequate supply of carbon source (i.e., acetate) for *Dehalococcoides* organisms. Only acetate (3.4 ± 0.2 mM) and propionate (3.10 ± 0.05 mM) were detected in the positive control cultures. Electron donor (hydrogen) was added in excess and, therefore, was not limiting. Despite the lack of dechlorination activity, *Dehalococcoides* organisms were easily detected in the oxygen-amended cultures, although cell numbers declined nearly 5-fold from 7.9 ± 0.5 x 10^6 to 1.7 ± 0.2 x 10^6 per mL during the 30 day incubation (Figure 8.1B). The three reductive dehalogenase genes *vcrA*, *tceA*, and *bvcA* were also easily quantified in the oxygen-amended cultures. Initially, the fractions of the total *Dehalococcoides* organisms present in BDI containing the *vcrA*, *tceA*, or *bvcA* genes were 82 ± 8 %, 18 ± 1 %, and <0.1%, respectively. Copy numbers of each reductive dehalogenase gene declined proportionally to the decrease in the total number of *Dehalococcoides* cells (i.e., about 5-fold reduction), resulting in identical proportions at the end of the incubation. For the control cultures incubated without oxygen, growth of the *Dehalococcoides* population was observed during VC dechlorination to ethene (Figure 8.1B). *Dehalococcoides* cell numbers increased from 7.9 ± 0.5 x 10^6 to 4.8 ± 0.6 x 10^7 per mL during the 30 day incubation (Figure 8.1B). The proportion of the *Dehalococcoides* organisms possessing the VC reductive dehalogenase gene, *vcrA*, increased from 82 ± 8 % to 95 ± 4 % during VC dechlorination. The percentage of the *Dehalococcoides* population possessing *tceA* declined from 18 ± 1 % to 5.3 ± 0.5 %. The copy number of *bvcA* increased by approximately 3-fold during the incubation, but the proportion of *Dehalococcoides*

organisms containing *bvcA* still represented <0.1 % of the total number of *Dehalococcoides* cells.

In an independent experiment, oxygen-amended BDI cultures failed to dechlorinate VC or produce ethene during 21 days of incubation (data not shown). In contrast, the oxygen-free control cultures dechlorinated VC and produced ethene. The *Dehalococcoides* cell numbers increased (e.g., growth occurred) in the control cultures. *Dehalococcoides* organisms, although inactive, were again easily detected in the oxygen-amended cultures. In contrast to the previous experiment, the number of *Dehalococcoides* cells in the oxygen-amended cultures remained virtually unchanged throughout the 21-day incubation.

In the TCE-fed cultures, the BDI consortium dechlorinated TCE to stoichiometric amounts of ethene in the absence of oxygen (Figure 8.2A). Initially, amendment of oxygen inhibited TCE dechlorination, but detection of *cis*-DCE on Day 30 indicated resumed dechlorination activity (Figure 8.2A). In fact, complete dechlorination of TCE to VC occurred by Day 60, and by Day 89, a small amount of ethene (5 mol% of the total chlorinated ethenes and ethene) was detected in the oxygen-amended cultures. As observed in the VC-fed cultures, the medium was initially pink in the TCE-fed, oxygen-amended vials; the medium turned colorless and then slightly turbid by Day 30 of the incubation. The concentrations of lactate fermentation products (e.g., acetate and propionate) were similar to those described above for the VC-fed cultures, again indicating *Dehalococcoides* organisms were not carbon source limited. Electron donor (hydrogen) was added in excess and, therefore, was not limiting. Although *Dehalococcoides* activity was severely inhibited, *Dehalococcoides* cells were easily

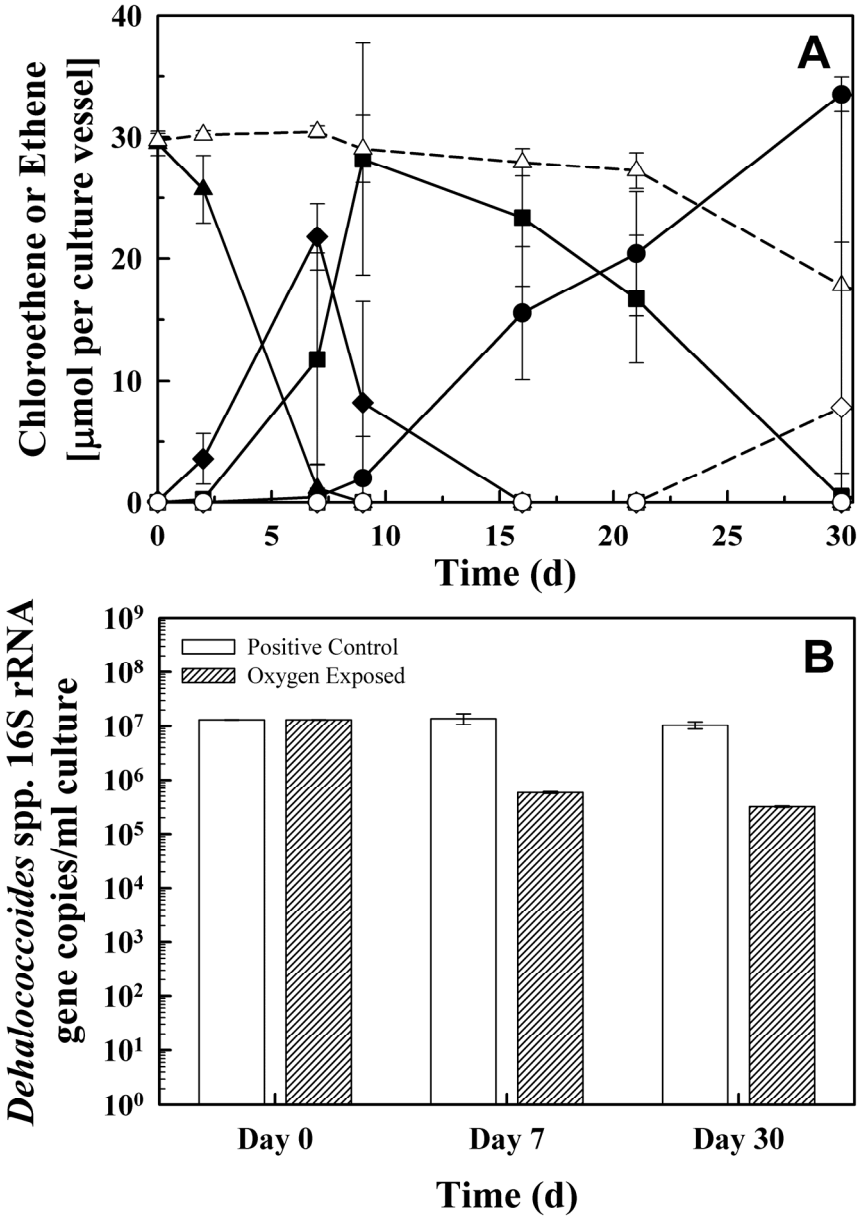

Figure 8.2 Dechlorination performance and qPCR analysis of the TCE-fed BDI consortium. (A) Dechlorination performance in the oxygen-amended (open symbols, dashed lines) or positive control (closed symbols, solid lines) cultures. Symbols: ▲, TCE; ◆, *cis*-DCE; ■, VC; ●, ethene. (B) qPCR analysis of *Dehalococcoides* spp. 16S rRNA gene copy numbers in the oxygen-amended and positive control cultures. All data points represent average values from triplicate cultures, and error bars represent one standard deviation. When error bars are not visible, they are smaller than and therefore hidden behind the data symbols.

detected in the oxygen-amended cultures. *Dehalococcoides* cell numbers decreased from $1.31 \pm 0.02 \times 10^7$ to $3.2 \pm 0.1 \times 10^5$ per mL in the oxygen-amended cultures during the 30 day incubation (Figure 8.2B). Similar decreases were also observed in the number of reductive dehalogenase gene copies (i.e., *bvcA*, *tceA*, and *vcrA*), although the reductive dehalogenase genes were still easily quantified via qPCR analysis.

8.4.2 Detection of Gene Transcripts During Oxygen Exposure

Transcripts of the *Dehalococcoides* 16S rRNA, *tceA*, and *vcrA* genes were detected in samples of the BDI stock culture used as inoculum for the oxygen-amended and control cultures. *bvcA* gene transcripts were not detected in samples from the stock culture, a likely consequence of the low RNA recovery (9.8 ± 0.9 %; see Materials and Methods section) and the small *bvcA*-containing *Dehalococcoides* population in BDI (<0.1 %). At the time of inoculation, the actively dechlorinating stock culture had completely dechlorinated its most recent amendment of TCE to *cis*-DCE, VC, and ethene. Therefore, detection of *tceA* and *vcrA* gene transcripts in the stock culture of BDI correlated with the concurrent presence of each reductive dehalogenases' substrate(s) (i.e., TCE and *cis*-DCE for TceA (*11*); *cis*-DCE and VC for VcrA (*6,13*)).

As in the stock culture, substrate-dependent transcription of the *tceA* and *vcrA* genes was observed in the oxygen-free, VC- and TCE-fed control cultures (see Figure 8.3A). Figure 8.3A presents the relative change in the number of transcripts per cell with time (i.e., the ratio of transcript copy numbers per cell to the number of transcript copies per cell in the inoculum). A ratio near unity (represented as 0 on the log scale in Figure 8.3A) indicates an insignificant change in the number of transcripts per cell. A ratio

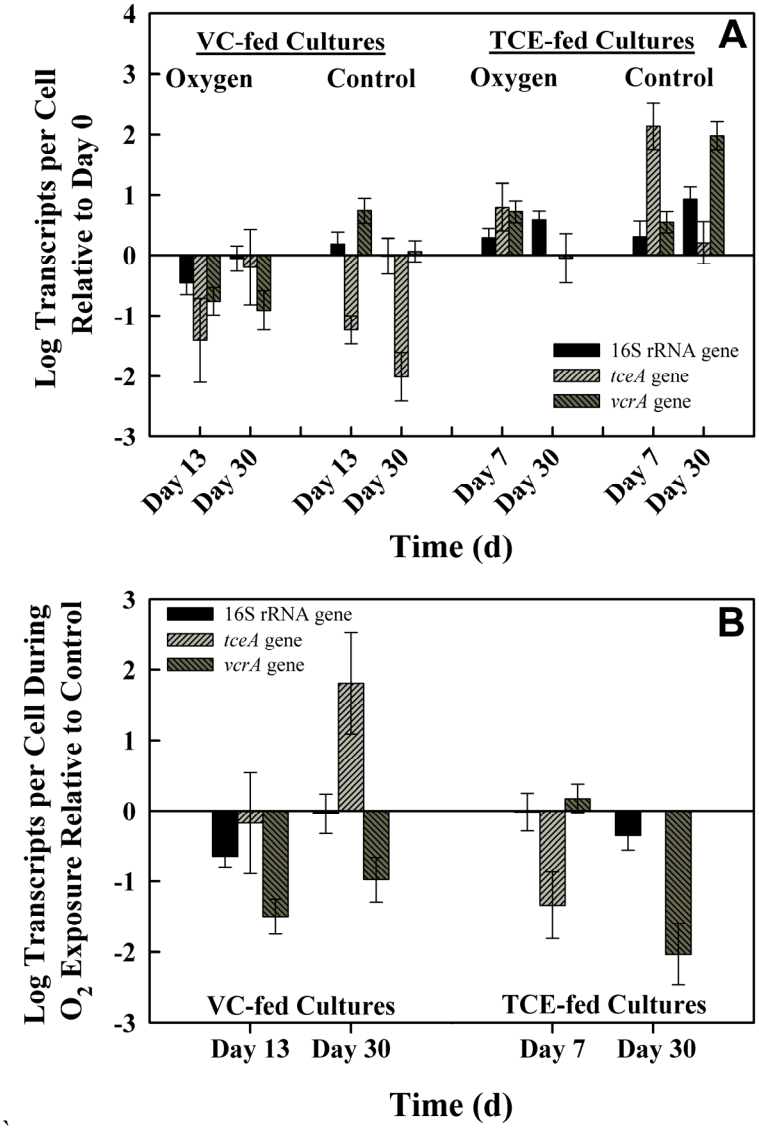

Figure 8.3 Reverse transcription (RT) qPCR analysis of *Dehalococcoides* 16S rRNA, *tceA*, and *vcrA* gene transcript copy numbers in the oxygen-amended and control BDI cultures. Transcript copy numbers are normalized on a per cell basis (e.g., *tceA* gene transcripts divided by genomic *tceA* gene copies). (A) Change in the number of transcript copies per cell in the oxygen-amended and positive control cultures relative to the number of transcript copies per cell in the inoculum (i.e., Day 0). (B) Change in the number of transcript copies per cell in the oxygen-amended cultures relative to the number of transcript copies in the positive control cultures. All data points represent average values from triplicate cultures, and error bars represent one standard deviation. *tceA* gene transcript copies were not detected in the oxygen-amended, TCE-fed cultures on Day 30; the lack of a bar in (A) and (B) for this time point, therefore, does not indicate a 1:1 ratio.

above unity (a positive number on the log scale) represents upregulation of gene transcription, while a ratio below unity (a negative number on the log scale) indicates downregulation of gene transcription. As shown in Figure 8.3A, transcription of the *tceA* gene was downregulated by 1.2 ± 0.2 to 2.0 ± 0.4 orders-of-magnitude in the VC-fed control cultures (Days 13 and 30, respectively). In contrast, the number of *vcrA* gene transcripts per cell in the VC-fed control cultures increased by 0.7 ± 0.2 orders-of-magnitude (Figure 8.3A, Day 13) before returning to levels similar to those observed initially. In the TCE-fed control cultures, transcription of the *tceA* gene was initial upregulated (2.1 ± 0.4 orders-of-magnitude; Figure 8.3A, Day 7) during TCE conversion to *cis*-DCE and VC (see Figure 8.2A, Day 7). By the end of the incubation, the number of *tceA* gene transcripts per cell returned to levels similar to those observed initially at Day 0 (Figure 8.3A). Transcription of the *vcrA* gene in the TCE-fed control cultures was upregulated at the onset of ethene production (0.5 ± 0.2 orders-of-magnitude; see Figures 8.2A and 8.3A, Day 7) and when VC conversion to ethene was nearing completion (2.0 ± 0.2 orders-of magnitude; see Figures 8.2A and 8.3A, Day 30). As with the stock culture of BDI, *bvcA* gene transcripts were not detected in the control cultures.

Surprisingly, transcripts of *Dehalococcoides* 16S rRNA, *tceA*, and *vcrA* genes, but not the *bvcA* gene, were also detected in the oxygen-amended VC- and TCE-fed cultures (Figure 8.3A). In contrast to the control cultures, transcription of the *tceA* and *vcrA* genes did not correlate to dechlorination activity and seemed independent of the presence and/or absence of specific electron acceptors (Figure 8.3A). In the oxygen-amended, VC-fed cultures, transcription of the target genes was downregulated (0.05 ± 0.21 to 1.4 ± 0.7 orders-of-magnitude; Figure 8.3A). For two of the samples from the oxygen-

amended, VC-fed cultures, the numbers of transcripts per cell were almost identical to those observed initially. Interestingly, the number of 16S rRNA, *tceA*, and *vcrA* gene transcripts per cell generally increased in the oxygen-amended, TCE-fed cultures (Figure 8.3A), although *tceA* gene transcripts were not detected on Day 30. The gene transcript copy numbers per cell were generally lower in the oxygen-amended cultures relative to the control cultures (Figure 8.3B). In several instances, the number of gene transcripts per cell in the inactive (i.e., oxygen-amended) and actively dechlorinating control cultures were indistinguishable (Figure 8.3B). In the VC-fed cultures, *tceA* gene transcripts per cell were actually detected at significantly higher levels (1.8 ± 0.7 orders-of-magnitude) in the oxygen-amended as compared to the control cultures (Figure 8.3B, Day 30).

8.4.3 Reversibility Experiments

Washed cell suspensions from oxygen-amended, VC-fed BDI cultures failed to dechlorinate VC in oxygen-free medium even after 161 days of incubation (Figure 8.4A). Similar data were observed in two of three triplicate cultures initiated by transferring aqueous samples from oxygen-amended cultures to oxygen-free medium (Figure 8.4B). In contrast, the third culture of the triplicates resumed VC dechlorination activity on Day 40 and slowly dechlorinated VC to ethene (Figure 8.4B). Additional samples taken on Day 262 revealed complete conversion of VC to ethene in the third culture of the triplicates, but ethene was still not detected in any other VC-fed replicate cultures. With washed cell suspensions (Figure 8.4A) or aqueous phase transfers (Figure 8.4B) from oxygen-free control cultures, dechlorination activity was observed by Day 10 and

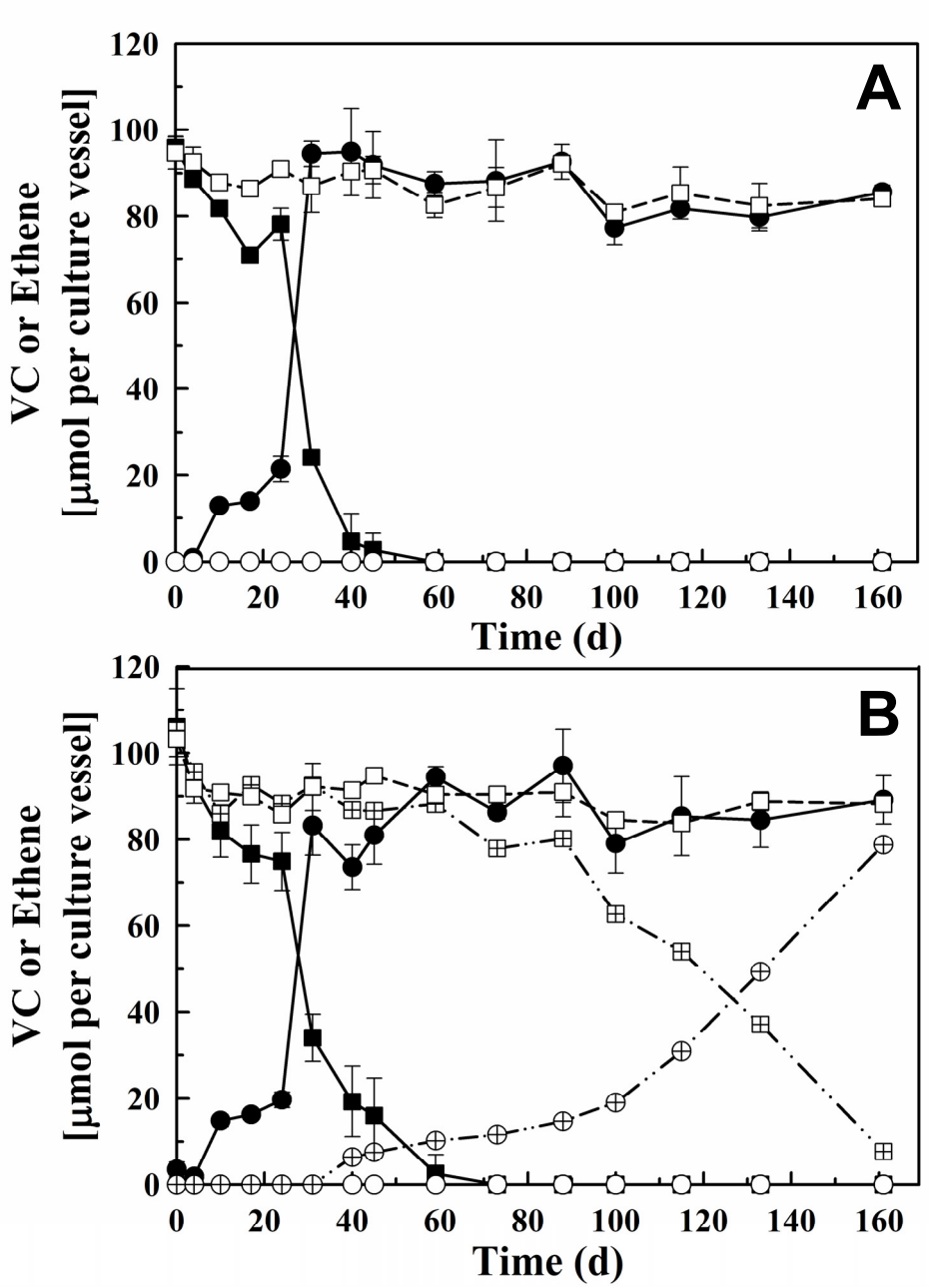

Figure 8.4 Reversibility experiments for the VC-fed BDI consortium with (A) washed cell suspensions or (B) 1% (vol/vol) transfers in identical sets of fresh, O_2-free medium. The inocula came from oxygen-amended (open symbols, dashed lines) or positive control (closed symbols, solid lines) cultures (see Figure 8.1). Symbols: ■, VC; ●, ethene. The closed symbols in (A) represent average values from duplicate cultures. The open symbols in (B) represent average values from two of three triplicate cultures, while the open, crossed symbols in (B) represent values from the third triplicate culture. All other data points represent average values from triplicate cultures. Error bars represent one standard deviation.

resulted in stoichiometric conversion of VC to ethene. Sufficient amounts (1.4 – 3.6 mM) of acetate were produced during lactate fermentation to serve as the carbon source for *Dehalococcoides* organisms in the VC-fed reversibility experiments. When an independent reversibility experiment was performed with washed cell suspensions from the second set of oxygen-amended, VC-fed BDI cultures, VC dechlorination and ethene production did not occur in oxygen-free medium during the 73-day incubation. In contrast, washed cell suspensions from oxygen-free control cultures dechlorinated VC and concomitantly produced ethene.

In oxygen-free medium, washed cell suspensions derived from TCE-fed control cultures dechlorinated TCE to stoichiometric amounts of ethene with intermediate formation of *cis*-DCE and VC (Figure 8.5A). In contrast, washed cell suspensions from oxygen-amended cultures dechlorinated TCE to VC with intermediate formation of *cis*-DCE (Figure 8.5B). Slow dechlorination of VC began on Day 31, after complete conversion of TCE and *cis*-DCE, resulting in gradual accumulation of ethene (22 mol% of the total chlorinated ethenes and ethene on Day 161) in the washed cell suspensions from the oxygen-amended cultures (Figure 8.5B). Similar data were observed in the reversibility experiments initiated by transferring aqueous phase samples from control cultures (Figure 8.5C) or oxygen-amended cultures (Figure 8.5D) to oxygen-free medium. In the aqueous phase transfer from oxygen-amended cultures, ethene slowly accumulated to 14 mol% by Day 161 (Figure 8.5D). Additional samples taken on Day 262 revealed continued, but slow, accumulation of ethene to 34 mol% and 23 mol% in the reversibility experiments with the washed cell suspensions and aqueous phase transfers, respectively. Acetate concentrations in the TCE-fed reversibility experiments

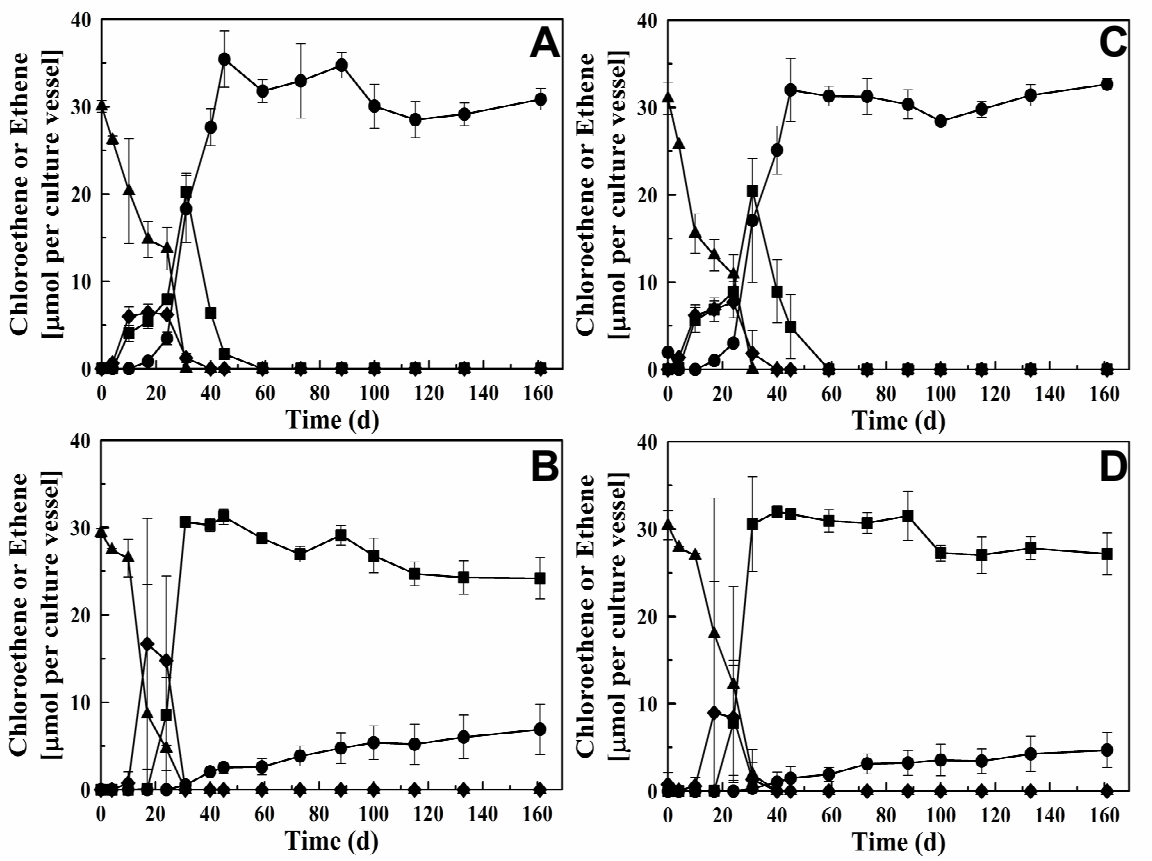

Figure 8.5: Reversibility experiments for the TCE-fed BDI consortium in identical sets of fresh, O_2-free medium. Inocula for the reversibility experiments came from oxygen-amended or positive control cultures (see Figure 8.2): (A) washed cell suspensions from positive control cultures; (B) washed cell suspensions from oxygen-amended cultures; (C) 1% (vol/vol) transfers from positive control cultures; (D) 1% (vol/vol) transfers from oxygen-amended cultures. Symbols: ▲, TCE; ◆, *cis*-DCE; ■, VC; ●, ethene. All data points represent average values from triplicate cultures. Error bars represent one standard deviation. When error bars are not visible, they are smaller than and therefore hidden behind the data symbols.

were similar to those described above for the VC-fed reversibility experiments, again indicating adequate supply of carbon source for *Dehalococcoides* organisms.

8.5 Discussion

Amendment of oxygen to the BDI consortium significantly, and sometimes irreversibly, inhibited the ability of *Dehalococcoides* organisms to reductively dechlorinate chlorinated ethenes. These results are consistent with previous observations that irreversible inhibition of pure cultures of *Dehalococcoides* spp. strains 195 and CBDB1 occurs after short exposure (~5 seconds) to air (*23*). The carbon source (i.e., acetate) for *Dehalococcoides* organisms was not directly supplied in the present experiments, but organic acid analysis revealed lactate fermentation to acetate in the oxygen-amended cultures, suggesting that the observed inhibition was not due to carbon source limitation but directly linked to the oxygen amendment. Oxygen levels were not quantified and likely declined before the initiation (i.e., inoculation) and/or duration of the experiment. Consumption of oxygen potentially occurred due to biological processes and/or abiotic reaction with chemical reductants (e.g., Na_2S) in the medium. Such processes may have consumed some or all of the amended oxygen, decreasing the duration of oxygen exposure or limiting contact of *Dehalococcoides* cells to oxygen. The observed inhibition of *Dehalococcoides* organisms, therefore, may not be due to direct interaction of oxygen molecules with *Dehalococcoides* cells. Alternatively, oxidation of the reductants may have resulted in elevated redox potentials above the optimum range for growth and activity of *Dehalococcoides* spp. Redox potentials greater than -100 mV

inhibit many anaerobic microbial processes (*30*). In the present study, the reduced medium turned pink (i.e., the redox indicator resazurin became oxidized) after oxygen addition, indicating that the redox potential of the medium was above -110 mV at the onset of the experiment (*27-30*). Although the oxidation state (i.e., color) of resazurin indicates redox potential, the indicator does not necessarily signify the presence or absence of oxygen. For example, low concentrations of oxygen can theoretically be present at redox potentials at which resazurin is colorless (*35*), and detectable levels of oxygen have been measured experimentally at similar redox potentials (-110 to -250 mV; (*36*)). Given that the direct role of oxygen in the observed inhibition of *Dehalococcoides* organisms is unclear, future research is needed that i) accurately quantifies oxygen levels and ii) evaluates the duration of *Dehalococcoides* exposure to and the kinetics of *Dehalococcoides* inactivation by various levels of oxygen. These studies should also explore *Dehalococcoides* inhibition at redox potentials elevated without the addition of oxygen.

Although the mode of *Dehalococcoides* inhibition observed herein is uncertain, quantifiable effects on *Dehalococcoides* cell numbers and activity were observed. In fact, the results of this study suggest i) a shift in the active dechlorinating population in BDI and ii) strain-specific differences in the ability of *Dehalococcoides* cells to remain viable following the oxygen amendment. Several lines of evidence suggest that, of the three *Dehalococcoides* strains in BDI, only strain FL2 survived the oxygen exposure. For example, TCE was dechlorinated to VC and traces of ethene in the TCE-fed reversibility experiments. Similar results were observed in the TCE-fed, oxygen-amended cultures after the redox potential fell below -110 mV (i.e., resazurin became

colorless) due to abiotic and/or biotic processes (see above). These results, coupled with the fact that rates of TCE dechlorination to VC were similar in the reversibility experiments initiated from the positive control and oxygen-amended cultures, suggest that the observed inhibition was reversible for TCE-to-VC dechlorinating *Dehalococcoides* organisms in BDI (i.e., strain FL2). Observation of slight, but incomplete, accumulation of ethene in the TCE-fed reversibility cultures is consistent with slow, cometabolic production of ethene, as observed in pure culture experiments with strain FL2 (*4*). In contrast to strain FL2, isolates GT and BAV1 metabolically dechlorinate VC to ethene (*3,6*). The results presented in this study suggest that these strains were not only inactive in the oxygen-amended BDI cultures but were also irreversibly inhibited. Only in one of eight VC-fed reversibility cultures was VC slowly dechlorinated to ethene after a significant lag time. One possible explanation for this observation is that a small fraction of VC-dechlorinating *Dehalococcoides* cells recovered after the oxygen amendment. Metabolic VC dechlorination was not observed in any of the other VC- or TCE-fed cultures, even after extended incubation (up to 262 days), suggesting that the observed recovery in the single culture was likely an experimental artifact (e.g., cross-contamination). These observations suggest that strain FL2-like organisms may be more robust and less sensitive to environmental stressors (e.g., exposure to oxygen) than VC-dechlorinating *Dehalococcoides* strains (e.g., strains GT and BAV1). Similar results were observed with *Dehalococcoides*-containing dechlorinating consortia exposed to elevated temperatures (Fletcher, Ramaswamy, Löffler, and Pennell; unpublished data), corroborating that strain FL2-like organisms are more tolerant of environmental stressors. Future studies should consider potential

interactions of *Dehalococcoides* with other bacteria (e.g., formation of cell aggregates); such interactions within complex microbial communities may help shield and protect strain FL2 (and potentially other *Dehalococcoides* strains) from exposure to and interaction with oxygen or other environmental stressors (*24,34*). Survival of strain FL2-like organisms, but not VC-dechlorinating *Dehalococcoides* strains, might lead to accumulation of VC during natural attenuation and bioremediation of contaminated sites. Such accumulation is undesirable but often occurs (*37*). Therefore, care should be taken during implementation of anaerobic bioremediation to i) limit exposure of bioaugmentation cultures to air, ii) deoxygenate remedial fluids (e.g., electron donor solutions), and iii) prevent oxygenated groundwater from reaching and influencing bioactive remedial zones (i.e., biobarriers).

Although the present study suggests strain-specific differences in the ability to tolerate and recover from oxygen exposure, current PCR-based tools used to detect and quantify *Dehalococcoides* DNA and RNA biomarkers did not prove useful in distinguishing viable, dechlorinating cells from inactive and irreversibly-inhibited cells. qPCR analysis easily detected high levels of *Dehalococcoides* DNA biomarkers (i.e., 16S rRNA and RDase genes) in the oxygen-amended cultures. In fact, the number of *Dehalococcoides* cells in one set of oxygen-amended cultures remained virtually unchanged throughout the incubation. Similar results were observed with another PCE-to-ethene dechlorinating consortium when exposed to oxygen (R. Daprato and J. Hughes, personal communication). Although decreases in the abundance of each DNA biomarker gene were observed in the two other sets of cultures, the biomarkers remained quantifiable, suggesting the potential for metabolic VC dechlorination. This conclusion,

however, is misleading since such activity was unrecoverable. Use of qPCR for detection of *Dehalococcoides* DNA biomarkers, therefore, has limitations in determining cell viability and predicting dechlorination activity.

Unlike DNA biomarkers, use of RNA biomarkers is generally considered to be a direct prognostic assessment of microbial activity as opposed to metabolic potential (*2,38*). Results presented herein suggest indicate that detection of *Dehalococcoides* RNA biomarkers (i.e., gene transcripts) via RT-qPCR does not always indicate active, dechlorinating cells. Interestingly, *Dehalococcoides* biomarker gene transcripts were detected in both the control and oxygen-amended cultures. In the control cultures, RDase gene transcription correlated to dechlorination activity and available chlorinated ethene(s). These results indicate the mRNA turnover occurred in the active cultures, which is consistent with previous studies (*39-41*). In the oxygen-amended cultures, RDase gene transcription did not correlate with dechlorination activity and appeared to be independent of substrate availability. RDase transcripts were detected in the oxygen-amended cultures, sometimes at levels similar to those in the control cultures, even when dechlorination was not occurring. In many cases, the number of biomarker gene transcripts per cell was indistinguishable in the inactive (i.e., oxygen-amended) and active (i.e., control) cultures. These results suggest that mRNA turnover (i.e., degradation) in inactive cells is slow; therefore, detection of RNA biomarkers may not directly indicate activity but instead represent "ghost" signals without correlation to metabolic activity. Alternatively, biomarker gene transcription might have occurred continuously in the oxygen-amended cultures. Future work should further investigate mRNA levels and turnover rates in actively dechlorinating, starved, and oxygen inhibited

cultures to improve application of RNA biomarker analysis to estimate *in situ* activity and dechlorination rates (*41*).

The use of PCR-based tools to assess and monitor bioremediation is gaining increased acceptance with regulators and environmental practitioners for analyzing the *Dehalococcoides* population (*2,38*). The results of this study suggest potential shortcomings in the resolution of these tools. New techniques (or improvements to existing ones) are needed to complement the current site assessment technologies. For example, recent studies in medical and food microbiology quantitatively distinguished viable and nonviable (dead) cells by pretreating samples with ethidium monoazide (EMA) or propidium monazide (PMA) before DNA extraction and qPCR analysis (*42-46*). EMA and PMA can penetrate "dead" cells with compromised cell membranes and cell wall systems. Covalent cross-linking of EMA or PMA with DNA by photoactivation prevents DNA extraction/purification and PCR amplification. Therefore, only DNA from viable cells can be detected after sample pretreatment with EMA or PMA. Such techniques, if applicable to *Dehalococcoides* organisms and *Dehalococcoides*-targeted DNA and RNA biomarker analysis, could easily be incorporated into existing protocols and commercially-available diagnostic services. Future work should also compare PCR-based tools to alternative approaches (e.g., proteomics, fluorescence *in situ* hybridization [FISH], compound-specific stable isotope analysis [CSIA]) as accurate measures of *in situ* microbial activity and cell viability. At present, the application of molecular biological tools to assess and monitor bioremediation has greatly improved the ability of scientists and engineers to establish cause-and-effect relationships between anaerobic microbial processes and contaminant detoxification (*2,9,38*). The potential limitations of

molecular tools described herein do not imply these approaches are ineffective, and the results of the present study should not be used to discount these valuable diagnostic technologies. Additional gains in refining *Dehalococcoides*-targeted biomarker analysis are likely to overcome such shortcomings, further enhancing the power of molecular tools for implementing reliable, cost-effective bioremediation of chlorinated ethene-impacted sites.

8.6 References

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CHAPTER 9

CONCLUSIONS AND RECOMMENDATIONS

Microbial reductive dechlorination (i.e., [de]chlororespiration) is emerging as a promising approach for the remediation of tetrachloroethene (PCE) dense nonaqueous phase liquid (DNAPL) source zones. For successful implementation of the microbial reductive dechlorination process to remediate DNAPL source zones, the mechanisms controlling microbial activity in the presence of DNAPL and the influence of microbial distribution on biologically-enhanced (bioenhanced) dissolution must be elucidated. Synergistic effects of coupling physical-chemical remedies with biological activity, as observed in recent pilot-scale field demonstrations of surfactant and cosolvent (ethanol) flushing, also need further exploration as a potential strategy for PCE-DNAPL source zone treatment. The overall objective of this research, therefore, was to address key gaps in the scientific and engineering understanding of PCE-DNAPL source zone bioremediation when applied in isolation or as a "polishing" step for the removal of residual DNAPL remaining after application of surfactant enhanced aquifer remediation (SEAR), an emerging physical-chemical treatment.

Experiments to assess microbial reductive dechlorination in the presence of PCE-DNAPL and at high dissolved-phase PCE concentrations expected near/in DNAPL source zones were performed with cultures of *Sulfurospirillum multivorans*, *Desulfuromonas michiganensis* strain BB1, *Geobacter lovleyi* strain SZ, and *Desulfitobacterium* sp. strain Viet1. Despite recent evidence suggesting that microbial reductive dechlorination occurs at or near PCE saturation (0.9 - 1.2 mM), all pure

cultures tested ceased dechlorinating at ~0.54 mM PCE. In the presence of PCE DNAPL, strain BB1 and strain SZ initially dechlorinated PCE, but dechlorination ceased when aqueous PCE concentrations reached inhibitory levels. For *S. multivorans*, dechlorination proceeded at a rate sufficient to maintain PCE concentrations below inhibitory levels, resulting in complete dissolution and transformation of the PCE DNAPL. These results (i) suggest that microorganisms incapable of dechlorinating at high PCE concentrations can enhance the dissolution of PCE from free-phase DNAPL under the appropriate environmental conditions and (ii) challenge the current paradigm that activity of dechlorinating microorganisms at high, saturated PCE concentrations is necessary for bioenhanced PCE-DNAPL dissolution. The results of this study, therefore, enhance the understanding of microbial species that can potentially play significant roles in bioenhanced dissolution.

Although several studies have evaluated source zone bioremediation, little is known about the contributions of individual dechlorinating populations to or the effect(s) of microbial distribution within the source zone on bioenhanced dissolution. Therefore, continuous-flow column experiments were performed to evaluate the distribution and activity of key dechlorinating populations within PCE-DNAPL source zones. Two cultures were used in the column experiments: a pure PCE-to-*cis*-DCE dechlorinating isolate, *S. multivorans*, as well as a robust PCE-to-ethene dechlorinating consortium, BDI-SZ, which contained multiple *Dehalococcoides* spp. and two PCE-to-*cis*-DCE dechlorinating populations (*Geobacter lovleyi* strain SZ and a *Dehalobacter* species). In a column containing pure PCE-DNAPL, *S. multivorans* failed to dechlorinate or enhance contaminant dissolution. The lack of activity and growth of *S. multivorans* in the

presence of pure PCE-DNAPL was attributed to inhibitory effects of high, saturating concentrations of PCE. The conditions within the pure PCE-DNAPL experiment represented a "worst case" scenario (i.e., the highest possible aqueous phase PCE concentrations). It is unlikely that PCE would uniformly reach such high concentrations throughout a real world DNAPL source zone. To better mimic heterogeneous source zones where dispersive transport and dilution processes may attenuate PCE to non-inhibitory levels, two additional column experiments (one with *S. multivorans* and one with BDI-SZ) were performed with mixed-NAPL (0.25/0.75 mol/mol PCE dissolved in hexadecane) source zones. In these experiments, spatial and temporal changes in the cell numbers of *S. multivorans*, *G. lovleyi*, and *Dehalococcoides* strains tracked closely with changes in the production of PCE dechlorination products. *Dehalobacter* organisms played a minor role in PCE dechlorination and bioenhanced dissolution in the BDI-SZ column experiment. The results from the mixed-NAPL column experiments indicate that some dechlorinating populations successfully colonize and are active within PCE-NAPL source zones. Microbial activity of *S. multivorans*, *G. lovleyi*, and *Dehalococcoides* strains resulted in significant bioenhanced PCE-NAPL dissolution, characterized by cumulative enhancement factors of 4.7 and 5.2 in the *S. multivorans* and BDI-SZ column experiments, respectively. These enhancement factors are comparable to those reported in the literature. Interestingly, results presented herein also suggest that source zone NAPL architecture (i.e., the presence of fingered NAPL regions in transition zones) affects bioenhanced dissolution. Despite the observed dissolution enhancements, PCE was not completely transformed to benign ethene in the BDI-SZ experiment, but stalled at *cis*-DCE and traces of VC. Accumulation of these toxic intermediates has also been

reported in other bioenhanced dissolution experiments. Incomplete dechlorination during the BDI-SZ column experiment is likely due to insufficient residence times and inhibitory levels of *cis*-DCE produced during the experiment (up to 2,600 μM). Declines in system pH due to the acidifying dechlorination process were also observed and may have contributed to incomplete dechlorination. Although complete detoxification of PCE to ethene is desirable, partial dechlorination is still beneficial in reducing source zone longevity. Alternative remediation strategies (e.g., coupling of physical-chemical with biological remedies) may help overcome the current shortcomings (e.g., incomplete dechlorination, inhibitory levels of polychlorinated ethenes) of source zone bioremediation.

One such alternative strategy is SEAR followed by microbial reductive dechlorination. Recently, synergistic effects of coupling these technologies were observed in a pilot-scale field demonstration at the Bachman Road site. For microbial reductive dechlorination to be considered feasible following SEAR, the surfactant(s) utilized during SEAR should have little or reversible impact (i.e., toxicity, inhibition) on the microbial populations relevant for the detoxification process. Therefore, experiments were performed to assess the possible inhibitory effects of Tween 80, a nonionic surfactant employed in source zone remediation, on microbial reductive dechlorination. Tween 80 did not inhibit dechlorination by PCE-to-*cis*-DCE or PCE-to-TCE dechlorinating cultures at surfactant concentrations similar to those observed at the Bachman Road site following SEAR (i.e., < 5,000 mg/L). In contrast, *cis*-DCE-dechlorinating *Dehalococcoides* isolates (strain BAV1 and FL2) failed to dechlorinate in the presence of Tween 80. Bio-Dechlor INOCULUM (BDI), a PCE-to-ethene

dechlorinating consortium, produced *cis*-DCE in the presence of Tween 80, further suggesting that Tween 80 inhibits dechlorination past *cis*-DCE by *Dehalococcoides* organisms. Quantitative real-time PCR analysis (qPCR) applied to BDI revealed that the number of *Dehalococcoides* cells decayed exponentially during Tween 80 exposure. Although Tween 80 exposure prevented ethene formation and reduced *Dehalococcoides* cell numbers, *Dehalococcoides* organisms remained viable, and dechlorination activity past *cis*-DCE was recovered following the removal of Tween 80. These findings suggest that sequential Tween 80 flushing followed by microbial reductive dechlorination is a promising strategy for remediation of chlorinated ethene-impacted source zones.

The application of molecular biological tools (e.g., qPCR) to assess and monitor bioremediation has greatly improved the ability of scientists and engineers to establish cause-and-effect relationships between anaerobic microbial processes and contaminant detoxification. In the present work, new molecular tools (i.e., qPCR protocols) were designed, optimized, and applied to specifically detect and quantify *S. multivorans* and *G. lovleyi* strain SZ. As described above, the use of these new molecular tools was critical in exploring the distribution of these environmentally-relevant dechlorinators within NAPL source zones. Based on the important role of *G. lovleyi* in bioenhanced dissolution in the BDI-SZ column experiment, an additional study evaluated (i) the environmental distribution (biogeography) of *G. lovleyi* and (ii) the ability of this versatile organism to respond to *in situ* biostimulation at a mixed waste site (Integrated Field-Scale Subsurface Research Challenge [IFC] site in Oak Ridge, Tennessee). Application of the specific molecular tools detected strain SZ-like amplicons in several PCE-dechlorinating enrichment cultures, the KB-1 bioaugmentation consortium, and in

environmental samples from chlorinated ethene-impacted aquifers. These results suggest that dechlorinating *Geobacter* spp. may be relevant contributors to chlorinated ethene detoxification in diverse environments. At the mixed (chlorinated solvent, nitrate, and uranium) waste Oak Ridge IFC site, the numbers of strain SZ-like cells increased from below detection to as high as $2.3 \pm 0.1 \times 10^7$ per liter groundwater following biostimulation, suggesting that strain SZ-like organisms contribute to contaminant transformation and, importantly, respond to *in situ* biostimulation. The new detection and quantification tools developed as part of this research add to the armamentarium of molecular tools available to remedial managers for site assessment and bioremediation monitoring. These tools may prove useful for evaluating source zone bioremediation as well as other biological remedies (i.e., biobarriers, monitored natural attenuation).

Strict anaerobic *Dehalococcoides* strains play critical roles in detoxification of chlorinated contaminants (e.g., chlorinated ethenes), and molecular tools that target *Dehalococcoides* DNA and RNA biomarkers are important in monitoring and assessing bioremediation (including source zone bioremediation). These strict anaerobes may be exposed to oxygen during: (i) transport and subsurface injection of *Dehalococcoides*-containing bioaugmentation cultures, (ii) subsurface delivery of electron donor(s) or other remedial solutions, and (iii) infiltration of oxygenated groundwater (e.g., rain events). The effect(s) of oxygen on *Dehalococcoides* activity and biomarker quantification are unknown. Therefore, experiments were performed to explore the effects of oxygen on *Dehalococcoides* viability and to evaluate the resolution of current PCR-based tools to distinguish active, dechlorinating cells from inactive, oxygen-exposed cells. The experiments were performed with BDI, a PCE-to-ethene dechlorinating consortium that

has been successfully used in bioaugmentation field applications and contains at least three *Dehalococcoides* strains (strains FL2, GT, and BAV1). Quantifiable effects on *Dehalococcoides* activity following oxygen exposure were observed. Several lines of evidence suggest that, of the three *Dehalococcoides* strains in BDI, only strain FL2 (a TCE-to-VC dechlorinator) survived oxygen exposure. Survival of strain FL2-like organisms, but not VC-dechlorinating *Dehalococcoides* strains (e.g., strains GT and BAV1), might lead to undesired accumulation of VC during natural attenuation or engineered bioremediation of contaminated (DNAPL) sites. Interestingly, the current PCR-based tools used to detect and quantify *Dehalococcoides* DNA and RNA biomarkers did not prove useful in distinguishing viable, dechlorinating cells from inactive and irreversibly-inhibited cells. The results of this study suggest that the current methodologies to detect and quantify *Dehalococcoides* DNA and RNA biomarkers have limitations in inferring cell viability and activity. New molecular tools are needed to complement existing technologies to improve the application of biomarker analysis in site assessment and bioremediation monitoring.

The research detailed in this dissertation explored the contributions of the microbial reductive dechlorination process (i.e., anaerobic bioremediation) to PCE-DNAPL source zone remediation. The results of this study suggest many areas of future research:

- Carefully conducted studies similar to those described in Chapter 3 should be performed with other PCE-dechlorinating bacteria to elucidate culture-specific inhibitory PCE concentrations.

- Since dispersive transport and dilution are relevant processes that may help attenuate PCE to non-inhibitory levels in source zones with heterogeneous NAPL distributions, additional work is needed to address the role of these processes in the performance of source zone bioremediation.
- At present, the complex interactions among dechlorinators and other microbial populations are poorly understood. Based on the research described herein, the following processes should be investigated:
  - Due to diffusional limitations and/or sorption processes, large cell aggregates that characterize some dechlorinating consortia may help protect dechlorinating organisms from environmental stressors, including elevated PCE concentrations, surfactants, or oxygen. Future research to understand the roles of complex microbial assemblages (i.e., cell aggregates, biofilms) in source zone bioremediation is needed.
  - Stress response systems are important for microbial tolerance to non-chlorinated solvents (e.g., toluene). Interspecies communication following stress exposure and the regulation of stress responses have been shown to result in stress-adapted phenotypes. Additional studies are needed to explore if such processes play roles in avoiding PCE inhibition and enhancing dechlorination activity in the presence of PCE DNAPL.
- At present, the factor(s) limiting complete detoxification (e.g., ethene formation) during source zone bioremediation are unknown. Future endeavors should systematically address the effects of system residence times, pH, and *cis*-DCE

concentrations on complete chlorinated ethene detoxification during bioenhanced dissolution.

- Evaluation of microbial distribution and bioenhanced DNAPL dissolution in more heterogeneous systems (e.g., 2-D aquifer cells) is needed, especially since the results presented herein suggest that source zone architecture (i.e., length, presence of transition zones) affects bioenhanced dissolution.
- Future research should evaluate the effects of environmental parameters, including surfactant concentrations, degradation, sorption, and dilution, on the pseudo first-order decay rate, *k*, that characterized the exponential decrease in *Dehalococcoides* cell numbers during Tween 80 exposure. Understanding environmental influence on *k* is critical to the incorporation of this parameter into remedial design models.
- To aid in surfactant selection for SEAR, the effects of other commonly used surfactants on key dechlorinators (e.g., *Dehalococcoides*) should be determined.
- The feasibility of the SEAR/microbial reductive dechlorination "treatment train" approach needs to be validated in controlled field studies that monitor the fate and transport of Tween 80; surfactant fermentation and the production of organic acids, alcohols, and hydrogen; and the activity, distribution, and abundance of key microorganisms during and after source zone flushing.
- Melting curve analysis proved to be a powerful diagnostic tool to verify target sequence amplification and distinguish closely related bacteria (*G. lovleyi* and *G. thiogenes*). Future work should explore the use of melting curve analysis as an additional tool to distinguish environmentally relevant bacteria and increase the level of resolution of the molecular toolkit available to environmental microbiologists.

- The development of qPCR protocols and other quantitative, nucleic acid-based tools should not only evaluate the specificity of the approach but also explore the effects of non-target DNA, including DNA with high sequence similarity to the target DNA sequence, on the accuracy of quantification.
- Future studies are needed to evaluate the kinetics of *Dehalococcoides* inactivation by various levels of oxygen.
- To improve the application of RNA biomarker analysis to estimate *in situ* activity and dechlorination rates, additional work should investigate mRNA levels/turnover rates in actively dechlorinating, starved, and oxygen inhibited *Dehalococcoides* cultures.
- Methods to quantitatively distinguish viable and nonviable (dead) cells during biomarker analysis should be explored.
- Alternative methods (e.g., proteomics, fluorescence *in situ* hybridization [FISH], compound-specific stable isotope analysis [CSIA]) should be systematically compared to PCR-based tools as accurate measures of *in situ* microbial activity and cell viability.

The findings of this research advanced the scientific understanding of the microbial reductive dechlorination process and are relevant to environmental remediation practitioners. The results of this study will aid in the design and successful implementation of source zone bioremediation. The gains made in understanding the complex interactions and processes involved in microbial reductive dechlorination and bioenhanced PCE-DNAPL dissolution will further promote source zone bioremediation as a viable technology for lasting and cost-effective remediation of DNAPL-impacted aquifers.

APPENDIX A

CALCULATION OF CUMULATIVE MASS RECOVERY AND MASS TRANSFER ENHANCEMENT FACTORS FOR THE COLUMN EXPERIMENTS

The cumulative mass recovery and mass transfer enhancement factors for the *Sulfurospirillum multivorans* column experiment containing the mixed-NAPL (Chapter 4) is described in this appendix. Similar calculations were performed for the pure PCE-DNAPL *S. multivorans* column experiment (Chapter 4) and for the BDI-SZ column experiment (Chapter 5).

Initial PCE Loading

The mixed NAPL was comprised of PCE (MW^{PCE} = 165.83 g/mol) and hexadecane (HD; MW^{HD} = 226.4 g/mol) at a final ratio of 0.25/0.75 (mol/mol). Therefore, the PCE mole fraction (X^{PCE}) in the mixed-NAPL was initially 0.25 and the HD mole fraction (X^{HD}) was initially 0.75. The density (ρ^{NAPL}) of the mixed-NAPL was determined experimentally to be 0.86 g/mL (Chapter 4). The volume of NAPL (V^{NAPL}) residing within the column following the NAPL imbibition was 14 mL (Chapter 4). The mass of NAPL ($Mass^{NAPL}$) added to the column was therefore:

$$Mass^{NAPL}(g) = V^{NAPL} \times \rho^{NAPL} = 14\,mL \times 0.86\,g/mL = 12\,g$$

A mass balance was performed on the mixed NAPL as follows:

$$Mass^{NAPL}(g) = Mass^{PCE}(g) + Mass^{HD}(g) = Mol^{PCE} MW^{PCE} + Mol^{HD} MW^{HD}$$

The total number of moles (Mol^{Total}) was equal to the sum of the number of moles of PCE and HD, and the number of moles of each NAPL component could be related to Mol^{Total} via the mole fraction (X):

$$Mol^{Total} = Mol^{PCE} + Mol^{HD}$$
$$Mol^{PCE} = X^{PCE} Mol^{Total}$$
$$Mol^{HD} = (1 - X^{PCE}) Mol^{Total} = X^{HD} Mol^{Total}$$

Making substitutions:

$$Mass^{NAPL}(g) = X^{PCE} Mol^{Total} MW^{PCE} + X^{HD} Mol^{Total} MW^{HD}$$
$$= \left(X^{PCE} MW^{PCE} + X^{HD} MW^{HD}\right) Mol^{Total}$$

Rearranging to solve for Mol^{Total}:

$$Mol^{Total} = \frac{Mass^{NAPL}(g)}{\left(X^{PCE} MW^{PCE} + X^{HD} MW^{HD}\right)} = \frac{12g}{\left(0.25(165.83g/mol) + 0.75(226.4g/mol)\right)}$$
$$Mol^{Total} = 0.05692\, mol$$

From above,

$$Mol^{PCE} = X^{PCE} Mol^{Total} = 0.25(0.05692) = 0.014231 mol = 14{,}231 \mu mol$$
$$Mol^{HD} = X^{HD} Mol^{Total} = 0.75(0.05692) = 0.04269 mol = 42{,}690 \mu mol$$

indicating that 14,231 μmol of PCE was initially added to the column in the mixed-NAPL.

Experimental Chlorinated Ethene Recovery

The recovery of chlorinated ethenes in the column effluent was determined by taking the area under each chlorinated ethene curve in Figure 4.3 for each component (PCE, TCE, *cis*-DCE, and *trans*-DCE) and then summing the mass recovery for each component to yield the total chlorinated ethene recovery. Mathematically, chlorinated ethene recovery was determined as described below.

For each effluent sample (x), mass recovery for component i ($MassRecovery_{i,x}$ where i = PCE, TCE, *cis*-DCE, or *trans*-DCE) was determined as follows:

$$MassRecovery_{i,x}\left(\mu mol\right) = \frac{C_{i,x}\left(\mu M\right) \times Q\left(mL/min\right) \times T_x\left(min\right)}{1000\,mL/L}$$

where $C_{i,x}$ is the experimentally measured concentration of component i in the effluent sample x, Q is the flow rate (0.25 mL/min), and T_x is the total time in minutes over which the effluent sample x is representative. For example, a sample was collected from the column effluent at x = 8.58 PV containing *cis*-DCE (3386.12 µM) and *trans*-DCE (19.19 µM) but not PCE or TCE (Figure 4.3). The sample prior to the 8.58 PV sample was collected at 7.67 PV and the next sample was collected at 9.54 PV. The time over which the 8.58 PV effluent sample was representative was assumed to begin halfway between the 7.67 PV and 8.58 PV samples (at 8.13 PV) and end halfway between the 8.58 PV and 9.54 PV samples (at 9.06 PV). Therefore, the sample at 8.58 PV was representative for 0.93 PV of flushing (9.06 PV minus 8.13 PV = 0.93 PV). Since one pore volume contained 397.3 mL and the flow rate was at 0.25 mL/min, the total time (T_x) over which the 8.58 PV effluent sample was representative was:

$$\begin{aligned} T_{8.58PV}\left(min\right) &= Sample\ PV \times \left(\frac{397.3\,mL}{one\ pore\ volume}\right) \div Q \\ &= 0.93\,PV \times \left(\frac{397.3\,mL}{one\ pore\ volume}\right) \div 0.25\,mL/min = 1478\,min \end{aligned}$$

Therefore, the recoveries of the components (i) for the 8.58 PV effluent sample were:

$$MassRecovery_{PCE,8.58PV}\,(\mu mol) = \frac{0\mu M \times 0.25 mL/min \times 1478 min}{1000 mL/L} = 0\mu mol$$

$$MassRecovery_{TCE,8.58PV}\,(\mu mol) = \frac{0\mu M \times 0.25 mL/min \times 1478 min}{1000 mL/L} = 0\mu mol$$

$$MassRecovery_{cis-DCE,8.58PV}\,(\mu mol) = \frac{3386.12\mu M \times 0.25 mL/min \times 1478 min}{1000 mL/L} = 1251\mu mol$$

$$MassRecovery_{trans-DCE,8.58PV}\,(\mu mol) = \frac{19.19\mu M \times 0.25 mL/min \times 1478 min}{1000 mL/L} = 7\mu mol$$

The total chlorinated ethene mass recovery for the 8.58 PV effluent sample was:

$$\begin{aligned} MassRecovery_{Total,8.58PV}\,(\mu mol) &= \sum_i MassRecovery_{i,8.58PV} \\ &= MassRecovery_{PCE,8.58PV} + MassRecovery_{TCE,8.58PV} \\ &\quad + MassRecovery_{cis-DCE,8.58PV} + MassRecovery_{trans-DCE,8.58PV} \end{aligned}$$

$$MassRecovery_{Total,8.58PV}\,(\mu mol) = 0 + 0 + 1251 + 7 = 1258\mu mol$$

Cumulative mass recoveries for each component ($MassRecovery_{i,Cumulative}$) and for all chlorinated ethenes (total = sum of cumulative mass recoveries for each component *i;* $MassRecovery_{Total,Cumulative}$) throughout the course of the column experiment were calculated by adding the mass recovery for each sample *x* to the recoveries determined for the previous samples (*x-1*, *x-2*, *x-3*…) as follows:

$$MassRecovery_{i,Cumulative}\,(\mu mol) = \sum_x MassRecovery_{i,x}$$

$$MassRecovery_{Total,Cumulative}\,(\mu mol) = \sum_x \sum_i MassRecovery_{i,x}$$

At the conclusion of the experiment (18.48 PV), cumulative mass recoveries for each component (*i*) were 75.8 µmol, 12.5 µmol, 7409.2 µmol, and 66.9 µmol for PCE, TCE,

cis-DCE, and *trans*-DCE, respectively. The cumulative mass recovery ($MassRecovery_{Total,Cumulative}$) for all chlorinated ethenes throughout the course of the experiment is shown in Figure 4.6A. At the conclusion of the experiment, the cumulative mass recovery ($MassRecovery_{Total,Cumulative}$) for all chlorinated ethenes was 7,564.4 μmol. Percent recoveries were calculated by dividing the cumulative recoveries by the total amount of PCE initially present in the residual NAPL source zone (14,231 μmol; see above).

Expected Abiotic PCE Recovery

The expected abiotic PCE recovery in the column effluent for each sampling event (x) was determined as follows:

$$ExpectedMassRecovery_{PCE,x}(\mu mol) = \frac{X_x^{PCE} \times C_{PCE}^{sol}(\mu M) \times Q(mL/min) \times T_x(min)}{1000\,mL/L}$$

where X_x^{PCE} is the mole fraction of PCE in the NAPL when the effluent sample x was collected, C_{PCE}^{sol} is the aqueous-phase equilibrium solubility of PCE from pure PCE DNAPL (1206 μM), and all other parameters are as described in the preceding section. This calculation assumes equilibrium mass transfer (Section 4.2.6) and the absence of bioenhanced dissolution (i.e., abiotic conditions). Initially, X_x^{PCE} was 0.25 (Chapter 4). X_x^{PCE} in the mixed-NAPL decreased throughout the experimental (biotic) column system due to PCE dissolution from the NAPL phase. The calculation of the expected abiotic PCE recovery accounted for the decrease in X_x^{PCE} in the experimental (biotic) column system as follows:

$$X_x^{PCE} = \frac{Mol_x^{PCE}}{Mol_x^{PCE} + Mol_x^{HD}} = \frac{Mol_{x=0}^{PCE} - \sum_x \sum_i MassRecovery_{i,x}}{\left(Mol_{x=0}^{PCE} - \sum_x \sum_i MassRecovery_{i,x} \right) + Mol_{x=0}^{HD}}$$

where Mol_x^{PCE} and Mol_x^{HD} are the total number of moles of PCE and HD residing within the mixed-NAPL when effluent sample *x* was collected, respectively, $Mol_{x=0}^{PCE}$ and $Mol_{x=0}^{HD}$ are the initial total number of moles of PCE (14,231 µmol) and HD (42,693 µmol) residing within the mixed-NAPL, respectively, and $\sum_x \sum_i MassRecovery_{i,x}$ is the cumulative recovery of all chlorinated ethenes (total = sum of cumulative mass recoveries for each component *i*) throughout the course of the experimental (biotic) column system up to the time when effluent sample *x* was collected. Mol_x^{HD} was assumed to be equal to $Mol_{x=0}^{HD}$ throughout the column experiment since HD is insoluble in water. An example calculation of X_x^{PCE} is demonstrated below for the conclusion of the biotic column experiment (18.48 PV) where the cumulative mass recovery was 7,564.4 µmol (see above):

$$X_{18.48PV}^{PCE} = \frac{Mol_{18.48PV}^{PCE}}{Mol_{18.48PV}^{PCE} + Mol_{18.48PV}^{HD}} = \frac{14{,}231 - 7{,}564.4}{(14{,}231 - 7{,}564.4) + 42{,}693} = 0.1351$$

Cumulative expected abiotic mass recovery for PCE throughout the course of the column experiment was calculated by adding the expected abiotic PCE recovery in the column effluent for each sampling event (*x*) to the expected recoveries determined for the previous samples (*x-1*, *x-2*, *x-3*…) as follows:

$$ExpectedMassRecovery_{PCE,Cumulative}\ (\mu mol) = \sum_x ExpectedMassRecovery_{PCE,x}$$

At the conclusion of the experiment (18.48 PV), cumulative expected abiotic PCE recovery ($ExpectedMassRecovery_{PCE,Cumulative}$) was 1,637.6 μmol. The cumulative expected abiotic PCE recovery throughout the course of the experiment is shown in Figure 4.6A. Percent recovery was calculated by dividing the cumulative expected abiotic PCE recovery by the total amount of PCE initially present in the residual NAPL source zone (14,231 μmol; see above).

Mass Transfer Enhancement Factors

A cumulative mass transfer enhancement factor was calculated by dividing the experimental cumulative mass recovery ($MassRecovery_{Total,Cumulative}$) by the cumulative expected abiotic PCE recovery ($ExpectedMassRecovery_{PCE,Cumulative}$):

$$Cumulative\ Mass\ Transfer\ Enhancement\ Factor = \frac{MassRecovery_{Total,Cumulative}}{ExpectedMassRecovery_{PCE,Cumulative}}$$

The cumulative mass transfer enhancement factor throughout the course of the column experiment is shown in Figure 4.6B. A cumulative mass transfer enhancement factor close to unity represents little bioenhanced dissolution. A cumulative mass transfer enhancement factor greater than one represents bioenhanced dissolution. At the conclusion of the experiment (18.48 PV), the cumulative mass transfer enhancement factor was 4.6, as shown below:

$$Cumulative\ Mass\ Transfer\ Enhancement\ Factor = \frac{MassRecovery_{Total,Cumulative}}{ExpectedMassRecovery_{PCE,Cumulative}} = \frac{7{,}564.4\,\mu mol}{1{,}637.6\,\mu mol} = 4.6$$

The cumulative mass transfer enhancement factor reached a maximum of 4.7 at 13.05 PV.

An alternative way of calculating mass transfer enhancement is by comparing experimental effluent total chlorinated ethene concentrations ($\sum_i C_{i,x}$) for effluent sample x to expected abiotic effluent concentrations ($X_x^{PCE} \times C_{PCE}^{sol}$). Enhancement factors calculated in this manner (termed maximum herein) are the ones most commonly reported in the literature and are always higher than cumulative enhancement factors.

$$Maximum\ Mass\ Transfer\ Enhancement\ Factor = \frac{\sum_i C_{i,x}}{X_x^{PCE} \times C_{PCE}^{sol}}$$

where $\sum_i C_{i,x}$ is the total chlorinated ethene concentration experimentally measured in effluent sample x, X_x^{PCE} is the mole fraction of PCE in the NAPL when the effluent sample x was collected, and C_{PCE}^{sol} is the aqueous-phase equilibrium solubility of PCE from pure PCE DNAPL (1206 µM). For example, a sample was collected from the column effluent at x = 8.58 PV containing *cis*-DCE (3386.12 µM) and *trans*-DCE (19.19 µM) but not PCE or TCE (Figure 4.3). Based on the calculated X_x^{PCE} at this point (0.207), the maximum mass transfer enhancement factor was:

$$Maximum\ Mass\ Transfer\ Enhancement\ Factor = \frac{\sum_i C_{i,x}}{X_x^{PCE} \times C_{PCE}^{sol}} = \frac{\sum_i C_{i,8.58}}{X_{8.58}^{PCE} \times C_{PCE}^{sol}}$$

$$= \frac{0\,\mu M + 0\,\mu M + 3386.12\,\mu M + 19.19\,\mu M}{0.207 \times 1206\,\mu M} = 13.6$$

VITA

Benjamin Keith Amos was born to parents Keith and Kathy Amos on December 26, 1978. He was raised in Sardis, Alabama, and was the valedictorian and class president of the 1997 graduating class of Sardis High School. Mr. Amos attended Auburn University to pursue his undergraduate degree. In May 2001, he received a Bachelor of Chemical Engineering *Summa Cum Laude* with the distinction of University Honors Scholar. He also completed internships at Southern Nuclear Operating Company in Birmingham, Alabama, and Oak Ridge National Laboratory in Oak Ridge, Tennessee, during his time at Auburn. Mr. Amos received a Master of Science from Georgia Tech in 2002, and then pursued doctoral studies in Environmental Engineering. He has received an NSF Graduate Research Fellowship, an EPA STAR Fellowship, and the Georgia Tech Institute Fellowship. During his tenure at Georgia Tech, Mr. Amos received several awards, including the 2005-2006 Civil and Environmental Engineering Bill Schutz Graduate Teaching Assistant Award and the 2006 Science Applications International Corporation (SAIC) Georgia Tech Student Paper Award. He was also named the 2007 Geosyntec Consultants Student Research Contest Winner and the 2005-2006 Outstanding Environmental Engineering Ph.D. Candidate. Mr. Amos volunteered at Westlake High School through the *Student and Teacher Enhancement Program* sponsored by Georgia Tech's *Center for the Enhancement of Teaching and Learning*. Upon completion of his doctoral degree, Mr. Amos intends to pursue an academic career and be involved in the education of future scientists and engineers.